SCIENCE FOR EVERYONE

N.N. BARASHKOV

LUMINESCENCE IN PUBLIC HEALTH

MIR

Science
for Everyone

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Люминесцентный анализ на службе здоровья

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Preface

This book introduces the reader to luminescence and its applications in biology and medicine. Luminescence methods have practically no rivals in medical and biological research from the point of view of availability of equipment, simplicity of manipulation, reproducibility of results, and sensitivity to small amounts of biological materials. Although these methods do not result in a qualitatively new stage in scientific knowledge, they do facilitate the resolution of a large number of problems existing today in medicine.

The volume of literature on the use of luminescence methods in health care has been increasing every year. However, their clinical application has been regretably small when compared to experimental studies. Thus we believe that this book based on accumulated experience has come into being as a helpful guide at an appropriate time.

When preparing the materials for the book the author was assisted by the researchers of the Biophysics Department of the Second

Moscow State Medical Institute and the Gamaleya Institute of Epidemiology and Microbiology. The studies carried out in these institutes are discussed here in more detail than those reported by other research centres. Because of the condensed volume of the book, it was impossible to dwell on all the studies that could be carried out using luminescence (fluorescence) analysis.

Introduction

Modern advances in medicine and biology have been made primarily owing to the use of analytical methods. Biologists were the first to use ultraviolet and infrared spectroscopy, mass spectrometry, radioactive analysis, and chromatography in their studies. Physicians and biologists need not only varied, but also highly sensitive methods of analysis, otherwise it is impossible to detect in the human body highly active drugs prescribed in minute doses. Recently, in medicine the nanogram (10^{-9} g) is more and more frequently used as a unit of mass. Such small quantities can be adequately detected by luminescence methods. This book considers the advantages of this method and its application in medicine and public health.

Luminescence and Luminophores

It is well known that heated objects emit light. This phenomenon is explained by the transformation of the energy of the thermal motion of atoms and molecules into the energy of light emission.

However, this has no relation to luminescence. Luminescence is any type of luminosity that is not connected with thermal light emission. Where do physical bodies obtain the energy necessary for luminescence? There exist several sources, i.e. the energy of chemical reactions (chemiluminescence) and of electric discharge (electroluminescence), and the energy of light absorbed by a body (photoluminescence).

According to the source of energy, all luminescent objects can be classified as chemiluminophores, electroluminophores, and photoluminophores. Further on we will deal only with photoluminophores, because they are the most important and widely used in practice. To become luminescent, a photoluminophore must be irradiated by ultraviolet rays (UV light), or short-wave rays of the visible part of the spectrum. As

a result, the molecules of the luminophore absorb energy and become excited. When they return to the ground state after a certain period of time, they emit a light quantum.

It is possible to observe quite a few natural luminophores around us. To illustrate their diversity, the following poetic description is given, although it was taken from a scientific monograph*:

"Just for a moment, let your imagination run free. Suppose that in the atmosphere surrounding the earth there is 'black gas' that completely absorbs the visible part of the light spectrum, but does not reduce the intensity of the UV rays reaching the earth's surface. Should this happen, an outlandish picture would appear before our eyes. The earth would be wrapped in complete darkness, but we would be able to discern a fairy tale world of luminescent objects. Without actually seeing the contours of the human body, we would see white flashes of teeth and blue fingernails. Many minerals would appear in different colours: violet fluorspar, red calcite, yellow orthoclase, etc. We might be attracted by the stunning beauty of a brown-grey stone with green spangles of uranium. We might notice the seeds of some plants, such

* Konstantinova-Shlezinger, M. A. (Ed.), *Luminescentnyi analiz* (Luminescence Analysis), Moscow, Fizmatgiz, 1961.

as vetch seeds, which would be coloured red, and seeds of oats that are differently coloured depending on their kind, etc. We would be surprised by the numberless multi-coloured sparkles on the meadows produced by parts of the flowers of many plants, e.g. the honey glandules at the base of the corolla.

"Sheets of white paper, depending on their quality, would appear blue, violet, or would be invisible. Spilled oil would look like dirty milk: black in daylight, in our imagined world it would have a yellowish-white glow. We might mistake kerosene or mineral oil stains for those of various shades of blue paint, because of their bright luminescence."

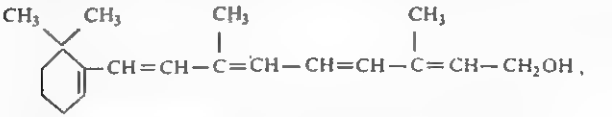
This excerpt describes natural luminophores, inorganic (minerals) as well as organic (seeds, flowers of plants, and oil products). The difference between these two types of luminophores is not only in the absence or presence of carbon in the molecule. There are a few more basic differences. Inorganic luminophores (which are sometimes called crystallophosphors) are luminescent only when in the solid state, this phenomenon being conditioned solely by a specific crystal lattice with certain activating admixtures. Organic luminophores are quite another case: here, separate molecules are luminescent, thus ensuring this phenomenon in the solid, liquid, and

gaseous states. We will consider mostly organic luminescence, because physicians and biologists most often deal with organic compounds.

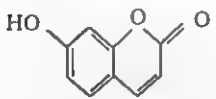
Depending on the duration of emission, one can distinguish two types of luminescence in organic substances: fluorescence, which ceases abruptly when the excitation source is removed, and phosphorescence, which persists for a certain time after the removal of the excitation source. In fact, this by far is not the only difference between fluorescence and phosphorescence. More essentially, light quanta in these processes are emitted from different energy levels of excited molecules.

What should the chemical composition of organic compounds be in order that they can luminesce? This question is not at all simple. Actually, the first serious investigations on the luminescence of organic substances were started after the publication of the classical work by the English physicist G. Stockes, which appeared in 1852. Although more than 130 years have passed, an adequate answer to this question has not yet been found. However, numerous observations have suggested that organic molecules with alternating single and double bonds between carbon atoms are luminescent more often. Chemists usually call this type of bonds conjugated, as well as the molecular structure of this type.

Natural luminescent compounds with conjugated structures are encountered in certain plants. These are, for example, vitamin A



and umbelliferone



When speaking of plant luminophores, the green leaf pigment chlorophyll cannot be overlooked. Its luminescence is dark red owing to the conjugated structure of the porphyrin ring contained in chlorophyll molecule. Given that the reader has already grasped the general idea of luminescence and organic luminophores, let us now examine the meaning of luminescence (fluorescence) analysis, which is used in the book. This term usually indicates the procedure of detecting and examining various objects and processes by means of fluorescence. This analysis consists in observing the light emitted by objects visually or by means of special devices. The high sensitivity of this method mentioned in the introduction is of great advantage here. In fact, it is

possible to work with extremely small amounts of luminophores (10^{-10} g per 1 g of substance being studied).

To get a clearer idea about just how small these figures are, imagine that every person of the 5 billion world population is going to be marked with a luminophore. It would turn out that only 0.50 g of a luminophore would be needed!

The theory of fluorescence analysis uses the concepts of the spectra of light absorption and fluorescence spectra of the substances studied. A typical pattern of these spectra (i.e. the dependence of the intensity of the absorbed and the emitted light on the wavelength) for complicated organic molecules is shown in Fig. 1. Examination of this figure reveals that luminescence includes two phenomena, namely fluorescence and phosphorescence. What is the difference between these two emissions? If the emission ceases immediately after the exciting light is removed, we call it fluorescence. But if an object remains luminescent for a certain period of time, then this is phosphorescence. However, there are other, more essential differences; the interested reader should see the comments to Fig. 1.

In this connection it is interesting to note, that if the spectra are represented as the intensity versus the wave frequency (the quantity that is the inverse of the wavelength), then the so-called rule of mirror

symmetry of absorption and fluorescence spectra can be observed for quite a few substances. According to this rule postulated

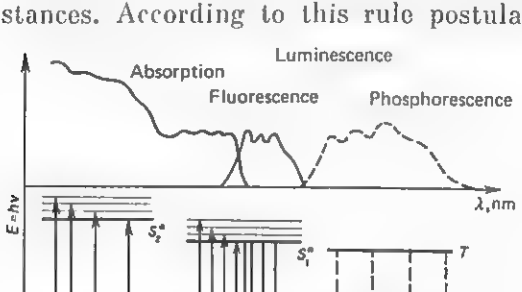


Fig. 1. Energy levels (below) and the spectra of absorption, fluorescence, and phosphorescence (above) of organic molecules.

At room temperature, virtually all molecules remain in the ground (non-excited) state (level S_0) After the absorption of a light quantum, a molecule becomes excited (levels S_1^* and S_2^*). This state of a molecule, when the spines of two non-paired electrons are antiparallel, is called the singlet state. Fluorescence is the emission of light during $S_1^* \rightarrow S_0$ electron transition. If the spines of the electrons within excited molecules are parallel, then these molecules are said to be in the triplet state (T-level). Phosphorescence is light emission during $T \rightarrow S_0$ transition. Phosphorescence spectra are closer to the violet region, as compared to fluorescence spectra

by the prominent Soviet physicist V.L. Levshin, absorption and fluorescence spectra are mirror images relative to the line perpendicular to the frequency axis and passing through the point of intersection of the spectral curves.

Now let us briefly touch upon the question of the energy of light absorption and emission by organic molecules. Of all the energy of incident light, only a small portion is actually absorbed by a molecule. Only a part of the absorbed energy is emitted in the form of luminescence; the rest is transformed into heat. Thus, luminescence is less bright than the exciting light. This relationship is characterized by the quantum efficiency of luminescence (φ), which is the ratio of the number of the light quanta emitted by a molecule (N_{lum}) to the number of the light quanta absorbed (N_{abs}) i.e. $\varphi = N_{lum}/N_{abs}$. The closer the value of φ to 1, the more intense is the luminescence of a particular molecule.

When performing quantitative luminescence measurements one should keep in mind the law postulated by the prominent Soviet scientist Academician S. I. Vavilov, which states that the quantum efficiency of luminescence and the form of luminescence spectrum for a particular substance do not depend on the wavelength of the exciting light.

Fluorescence analysis is easiest when the emission of the substance in question is very bright. Sometimes fluorescence analysis uses bright luminophores which are added to the substance under study. Where can such luminophores be found? Usually they have to be synthesized. When synthe-

sizing them, chemists are guided by the above-mentioned considerations about the conjugated structure of the luminophore molecules. A great variety of bright synthetic luminophores have already been produced. Later on, some of them will be named and, in some cases, their structural formulas given. In addition to intensity, luminophores must meet other requirements, necessary for their practical application, e.g. a specific colour of luminescence, solubility in various substances, stability to light and heat, chemical activity or inertness, etc.

Applications of Fluorescence Analysis

Before going into detail about the medical applications of fluorescence analysis, a brief account of the applications of fluorescence analysis in general science and technology should be given. Fluorescence analysis is most often employed when one wants to detect objects that are invisible in daylight. In paleontology, for example, fluorescence analysis is used to study the fossils of prehistoric plants and animals in sedimentary rock. When these fossils are illuminated by UV light, they begin to luminesce, and previously invisible details of the object structure can be thoroughly examined.

Luminescence is also invaluable in fault detection techniques, because one can see microcracks and microfissures that are undetectable by any other method. Though it differs slightly from that described above, this procedure is also quite simple. First, the machine components are coated with a luminophore solution which fills all the cracks and remains there after the coating layer is removed. Following this, all the faults are easily revealed by the UV light.

Back in the days of Sherlock Holmes, detectives were ignorant of the practical applications of luminescence. But modern criminologists have successfully made use of this phenomenon. Many illustrative examples can be given here. The natural luminescence of human skin can indicate the exact "age" of traumas (scars). The colour of natural luminescence of healthy skin is whitish-grey, whereas old scars reveal different colourings, i.e. a velvety dark colour 1-2 months after the trauma; whitish-blue with dark contours after 4-6 months; and, after a year or more, they give a whitish-bluish colour, or somewhat darker if the skin is pigmented.

Fluorescence analysis is essential for the accurate assessment of gunshot wounds. In fact, an inlet of any bullet can be detected by the blue luminescence of gun lubricant traces. When passing through the gun barrel, the bullet picks up traces of

lubricant which are left at the edges of the hole made by the bullet. After the lubricant traces are dissolved in ether and their luminescence intensity is measured, the successive order of the shots can be determined. This technique is still the most easily applicable and available one among those used in forensic medicine.

At present, luminescence is used to examine hair, for example, the original colour can be determined by the colour of glow of the cross section. Old blood stains can also be identified. The stain which is supposed to contain blood, is moistened with concentrated sulphuric acid; an orange-red glow indicates blood. Luminescence is also indispensable for determining time the bones were in earth; by the colour of the glow of the exhumed bones one can decide whether a corpse has been cremated or buried intact.

Luminophore powder can serve as a preventive, burglar-proof measure. The powder applied to the door knobs and handles of storerooms and safety boxes will label the hands of any intruder and will glow in UV light, thus providing an undeniable proof of guilt.

There are many other applications of fluorescence analysis. One of the simplest methods is the UV-examination of documents for determining which ones may have been forged. Under UV illumination, all the erased parts of the text reappear, as

well as glue and starch traces on forged seals.

The same method is also used by experts to reveal the author's signature or the traces of restoration. In 1928 it was proven that the author of the picture *The Heavy Sea* was not I. K. Aivazovsky, as was previously thought. Close examination outlined luminescent traces of another name beside the "signature" of the famous painter. In archaeological investigations UV light can restore vanished paragraphs in ancient manuscripts, as well as signatures in historic documents.

The method of luminophore labeling is quite popular in geology and hydraulic engineering. When projecting seaports, landing piers and dams, exact information about the direction and the rate of sand transfer within particular water reservoir is of paramount importance. This kind of information is provided by labeling sand with luminophores. Luminophore is dissolved in an aqueous solution of agar-agar and the obtained suspension is mixed with the sand. The mixture is then dried and a thin luminophore film sticks to the sand grains. This labeled sand is placed underwater for a certain period of time, after which sand samples are taken. Sampling is performed in various points of the reservoir, and by comparing the number of labeled particles in different samples, one can es-

tablish the pattern of sand transfer. This is a rather complicated, but reliable procedure.

The application of water-soluble luminophores in hydrogeology is much simpler. They are applied to measure reservoir capacities, river and sewerage flow rates, etc. For these purposes luminophores were employed long before radioactive isotopes came to the fore. In 1960 the bottom of the Baltimore Harbor (USA) was explored. The luminescent dye Rhodamine B was poured into the water in certain areas of the harbor. A specialized cruising ship was equipped with a fluorometer, i.e. a simple device for measuring the intensity of fluorescence of the water samples taken from a given depth. Thus, moving along a fixed route, the ship could collect data about the changes of the concentration of the luminophore in the water. The result was a detailed pattern of water circulation in the Baltimore Harbor.

Finally, it is hard to imagine the latest developments in analytical chemistry without fluorescence analysis. Fluorescence analysis can identify about 50 elements in the Mendeleev periodic table. The method can be applied even when the chemical compound in question does not luminesce itself. In this case it is necessary only to find a reagent that reacts with the compound, producing a luminescent substance.

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For example, the content of ozone in stratosphere samples was established by this method with high accuracy (the overall ozone content in the sample being less than 10^{-7} g). Luminescent indicators have been also widely used in titration, because they change the colour or intensity of the glow of the titrated solution at the equivalence point.

Applications of Luminescence in Medicine

The pioneering applications of fluorescence analysis were in the fields of medicine and biology. We have already mentioned the work by Stockes on the luminescence of organic compounds, which was published in 1852. The author gave a detailed account of emissions characteristic of quinine, chlorophyll and other luminescent substances of plant origin. For several decades fluorescence analysis has been used in the USSR and other countries to detect vitamins and traces of drugs, as well as for other similar purposes which will be dealt with further on. It might seem somewhat surprising that this method is still not used to the extent that it should be, because most of physiologically active compounds either luminesce, themselves, or can become luminescent after simple chemical treatment.

The radioactive isotope method is one of the few equally sensitive rivals of fluores-

cence technique. Pharmacologists, for example, can label the drug with a radioactive isotope and trace its path in the organism. However, this method is fairly expensive. The advantages of luminescence techniques are in their simplicity and availability, as well as in their complete harmlessness to the patient.

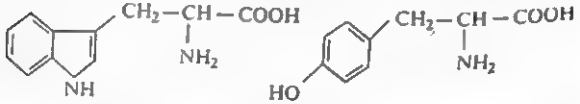
Medical investigations using fluorescence techniques fall into two large groups. The first group includes investigations of naturally luminescent objects, or objects acquiring this property after a certain change in their chemical structure. The second group makes use of luminophores specifically selected to label the objects under investigation. These luminophores are frequently called stains.

The two groups mentioned above differ considerably. When studying the natural luminescence of substances, they undergo no changes, and can be analyzed by other techniques. As for the second type of investigation, the luminescent stain can change the initial properties—a fact that must not be overlooked. But the latter is, of course, advantageous in other ways, e.g. the substance to be studied can be taken in very small amounts, because the glow of the stain is much brighter than that of the substance under investigation.

The stain must have the same properties as luminophores in general (see the preced-

ing section). It should be added that, apart from the bright glow, the spectral region of the luminescence of the stain ought to be well-defined and highly specific, so as to be able to distinguish its emission from the natural emission of the substance being studied. The stain should also tightly bind to the substance.

Proteins, which are most often the objects of medical luminescence studies, provide a vivid illustration of the above statements. Only few of them are naturally luminescent owing to the presence of aromatic amino acids such as tryptophan and tyrosine:

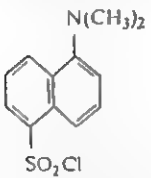


Among the naturally luminescent proteins there are bovine plasma albumin, tobacco mosaic viral protein, ribonuclease, and some others. Their emission is not intense and usually belongs to the UV region of the spectrum (up to 350 nm), i.e. beyond the range visible to the human eye, which is between 400 nm (violet rays) and 760 nm (red rays).

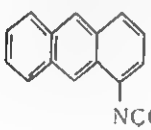
The measurement of the natural luminescence intensity underlies the quick and precise determination of protein content in milk. Mostly, however, researchers deal with non-luminescent proteins. It is

known that protein molecules contain various functional groups, such as $-NH_2$, $-NHR$, $-OH$, $-SH$, $-COOH$, and this has been taken advantage of by biologists and chemists for the luminescent staining of proteins.

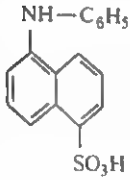
The staining procedure is quite uncomplicated. The proteins are reacted with the stain under conditions preventing protein denaturation. After the reaction is over, excess stain is carefully removed. This process may be either a chemical reaction, if the stain contains active groups that can bind to the functional groups of protein molecules, or an absorption process. For chemical reactions, specific stains are usually used that contain sulphochloride, isocyanate and other groups, such as dimethylaminenaphthalenesulphochloride (dansylchloride)



or anthraceneisocyanate



These compounds react mostly with the amino groups of proteins as a result of which sulphamide and urea bonds are formed. These formerly non-luminescent proteins acquire now a bright greenish glow. The luminescence of stained proteins is observable when there are 1-3 luminophore molecules per 1 protein molecule. The amount of dansylchloride and anthraceneisocyanate used is 100-200 times less than that of the protein to be stained, because these stains are usually applied for proteins with the molecular mass between 10 000 and 300 000. When the stain itself is intensely luminescent, it must be thoroughly removed from the stained protein. Still more advantageous appears to be the use of dye solutions that do not luminesce in the free state. Dansylchloride and sulfo acids of similar structure, like anilinenaphthalenesulfonic acid belong to this type of dyes:



This is also one of those substances that is bound to proteins by absorption forces rather than chemically. What then can account for the fact that anilinenaphthalenesulfo-

nic acid is luminescent only in the absorbed state? There is not clear answer to this question; however, investigators have offered a few explanations. In a solution, the molecules of a stain may have an irregular (nonplanar) configuration, which, according to the laws of quantum theory, prevents them from luminescing. When absorbed, these molecules acquire the planar configuration that makes luminescence possible.

Fluorescence analysis is particularly convenient in medical and biological investigations when the objects studied are very small in size. In fact the routine light microscope is unable to detect objects less than 0.2 μm. However, the organisms under investigations (e.g. viruses) may often be smaller in size. Therefore, the simplest way to visualize these objects is by staining them. Stained viruses may seem unchanged to the naked eye, but fluorescent microscope will distinguish them as glowing points against a dark background. The same is true for stars in the night sky, which can be seen only because of their light emission (the angular d ension of stars being many times less than the resolving power of human eye).

Various microscopic methods exist in luminescence investigations. They employ either transmitted light (in light and dark fields), or the light incident through the microscope lens. In fact, any light microscope

can be transformed into a fluorescent one by using the technique of "crossing" light filters (Fig. 2). Here, of course, only a sim-

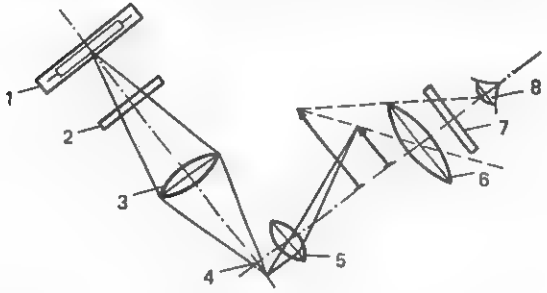


Fig. 2. A general scheme of a fluorescent microscope.

The light emitted by the source *1* (usually it is a mercury vapour lamp) goes through the light filter *2* which transmits only UV or blue rays; the transmitted light is then focused by the lens *3* upon the luminescent microobject *4*. The image of this microobject is magnified by means of an ordinary microscope for biological studies (*5*—lens, *6*—ocullar), and is transmitted through the filter *7* to the observer's eye or to the photocell *8*. The light filters *2* and *7* are "crossed", because the latter (usually amber) is completely impermeable to the light transmitted by the first filter

plified scheme is presented. For a more detailed illustration of a modern fluorescent microscope, see Fig. 3. In these microscopes, fluorescence is mostly excited by incident light transmitted through an epi-illuminator.

This luminescence excitation system was suggested in 1955 by the Soviet investi-

gator E.M. Broomberg. The principal detail within the epi-illuminator is an interference beam splitter coated with layers of dielectrics. By choosing the layers of dielectrics, one can obtain a beam splitter, which

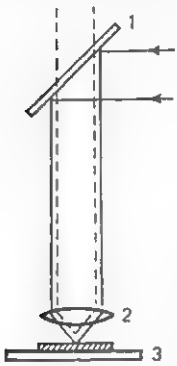


Fig. 3. A general scheme of epi-illuminator:
1—interference beam splitter; *2*—lens; *3*—object

selectively reflects up to 95% of light in one region of the spectrum and correspondingly transmits almost all the light in the other spectral region. The light reflected by the object, as well as by the glass and the microscope surfaces, is for the most part reflected by the splitter and does not reach the observer's eye. The advantages of the Broomberg system are obvious especially when using high-aperture lenses with a high degree of magnification, such as those applied in microbiological research. This system provides a maximum intensity of illumination for the object, making it pos-

sible to slightly change the angle between light filters. Thus, it is easy to examine thick and non-transparent objects as well as thin and transparent ones.

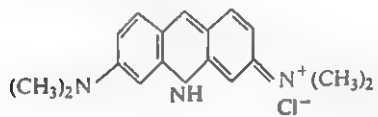
Such sophisticated fluorescent microscopes have been developed by Soviet industry. Fluorescent microscopes in which fluorescence is excited by the light incident through microscope lens using epi-illuminator with the interference beam splitter have only recently been manufactured abroad.

The latest МЛ-2 model simultaneously provides the luminescent picture (illumination from above) and the phase-contrast one (illumination from below) in the same field of vision, for the purpose of detecting non-stained areas in the specimen. This is the most universal microscope that can be used in laboratory investigations. In field conditions it is more convenient to work with the portable МЛД-1 model, which has amortization. Its stand compared to that of the MJI-2, is of simpler design, it has a smaller number of optical devices, and it cannot perform microphotography and phase-contrast examinations.

The first microscopic fluorescence study was performed in 1904 by the German scientist A. Köhler. Later, the famous Russian botanist M. S. Tsvett, the discoverer of chromatography, studied chlorophyll and cyanophyll in plant cells using

fluorescence microscopy. Further great progress was made by the German investigator M. Haitinger who, in 1933, provided a scientific basis for the selection and application of dyes for fluorescence microscopic studies. These substances, as a rule, bind to the specimen under investigation by means of absorption; they can selectively detect specific biological structures and are used as strongly diluted aqueous or alcohol solutions.

One of the most popular dyes, used in fluorescence microscopic studies is acridine orange



which possesses a peculiar feature of polychromatophilia. This term is used to characterize the ability of a dye to bind with different cellular structures and give differently coloured glow.

Sometimes the application of only one luminescent dye is not sufficient. In this case, two, three and even more different luminescent compounds are used that are differently absorbed by separate parts of a cell.

In 1952, Academician A. N. Nesmeyanov said that the application of fluorescence microscopy in medicine, biology and chemis-

try would open up such possibilities for scientific research which would have seemed unthinkable before. This idea can conclude this section. In later sections, in particular those dealing with various medical applications of fluorescence analysis, the validity of this suggestion will undoubtedly be demonstrated.

Investigations of Physiological Processes

For successful fluorescence observation of physiological changes in different organs of the human body, it is necessary to know the natural luminescence of these organs. In fact, all tissues and cells, with the small exception of haemoglobin and pigmented skin, are luminescent under UV light. Dermal and mucosal integument gives a blue luminescence whose intensity increases when sebaceous gland excretions are present. In general, the intensity of tissue luminescence is determined by the tissue structure and degree of haemoglobin saturation. The intensity of luminescence of dermal integument is accordingly dependent upon the blood level, the thickness of horny layer, age, etc. The luminescence of cartilage, bones, and teeth is much more intense than that of the skin, whereas the glow of muscles is hardly noticeable. The luminescence of bile is the most variable

in colour (from violet to red). The gums and the base of the tongue usually give dark red luminescence because of the presence of porphyrin derivatives. Dissected liver has an intense greenish-yellow glow.

What is then responsible for the natural luminescence of tissues and organs? Contemporary science holds the view that the colour and intensity of luminescence of biological systems are determined by the presence of luminophore compounds like porphyrin derivatives, which are responsible, for example, for the red glow of gums, as well as by chemiluminescent processes. A weak glow, especially in the green and blue spectral regions, can appear owing to oxidation reactions of lipid structures in the cell. This suggestion is supported by the fact that this glow diminishes with a decrease in the partial pressure of oxygen over living tissues.

The studies of chemiluminescence can yield valid information about the cell physiology, and damaged structures of a cell. It seems that weak chemiluminescence of biological objects is similar to that of ordinary chemical reactions proceeding with light emission, e.g. phosphorus oxidation. It was shown that the glow intensity of living tissues becomes visibly reduced after the addition of antioxidants, for example. cysteine. Another peculiar finding is that the intensity of chemiluminescence is independent of

the intensity of oxygen absorption by tissues. These data led to a suggestion that weak biological luminescence is, most probably, connected with non-enzymatic processes, including the oxidation of lipid structures, which do not directly participate in metabolic reactions.

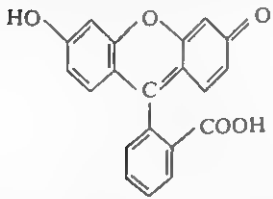
The nature of tissue chemiluminescence is further proved by the spectral similarities between emission occurring during spontaneous oxidation of separate lipids in the air and emission by the tissues of living organisms.

The above is characteristic only of healthy organs and tissues, whereas certain diseases are able to produce noticeable changes in their luminescence. Thus, an intense orange and red emission is characteristic of purulent tonsils and cholesteatoma*. The luminescent changes of the skin surface can detect eczemas long before they become visible to the eye. The appearance of brownish spots in the mouth cavity under UV light is a valid proof of jaundice (before the development of visible symptoms). Dental tissues affected by caries are not luminescent, as was demonstrated in 1957. This fact introduces another diagnos-

* Cholesteatoma is a tumour consisting of keratinized epithelial squamulas and developing in skin, bones and mucosa. Its surface is brightly white in colour, which accounts for its other name, "pearl tumour".

tic tool that could be used by dentists. The acute and chronic inflammatory processes in integuments cause a marked reduction in their luminescence intensity. These examples are numerous but, unfortunately, do not cover by far all the cases.

In medical examinations, specialists normally observe not the natural (inherent) luminescence, but the secondary luminescence emerging after luminophore staining. Human organs can be studied after intravenous or subcutaneous injection of luminophore solution. For this purpose fluorescein is used most often



Fluorescein has a bright emission and derives its name from the name of one of the types of luminescence—fluorescence. The advantage of fluorescein is that it is harmless to the organism (up to several grams). The intensity of the fluorescein emission is so high, that it can be detected in very small concentrations. For example, when a few millilitres of a concentrated solution is injected into the median cubital vein, the fluorescein spreads throughout the

entire body. In a few seconds, the lips, eyes and oral mucosa would noticeably luminesce with a green colour. This method is applicable for determining the time of blood circulation within the body, as well as areas of limited blood supply. In the forties, fluorescein was used for the first time in skin transplants. If the transplant became luminescent after a certain period of time, it was clear that circulation had been established between the host tissues and the graft. This method allowed for the early detection and prevention of post-operative skin necrosis in rejected areas.

Since 1958, fluorescein staining has been used for studying permeability of blood vessels in humans, and the effect of medicinal agents on this permeability. Fluorescein solution is also injected into the spinal fluid. This helps to detect spinal fluid that has penetrated into nasal cavity, middle ear and other organs. This is the only diagnostic method that can detect this type of affection. In addition, subcutaneous fluorescein injections are helpful in assessing hepatic and renal functional activity, as well as in neurological studies.

In 1969, the Swedish investigator K. Caspersson successfully demonstrated fluorescence analysis of amniotic fluid as a safe means of prenatal determination of sex. This kind of information is useful for many purposes, one of them being the prevention

of hereditary diseases such as haemophilia, i.e. severe bleeding resulting from the impairment of blood clotting processes, which is seen only in males. Such diseases are sex-dependent, i.e. the genes are passed on to all the progeny, but are active only in one sex. Studies of the luminescence of chromosomes stained with quinacrine hydrochloride revealed different intensity of chromosome parts. The male sex chromosomes (Y chromosomes) were brighter than the others, although the luminophore indiscriminantly stained all the chromosomes.

It must be noted that the luminescent parts of Y chromosome retain their emission within the nucleus of the cell before division, while the chromosomes themselves do not emit light. Every normal male cell stained with luminophore has a bright spot, while female cells have none. Long before these studies were performed, it had been shown that when the female non-dividing nuclei are stained with routine non-luminescent substances, the so-called Barr body, which is one of the two X chromosomes of the female cell, is revealed. When such stained bodies did not appear, the interphase cells were considered to be male. The fluorescence method serves as a valid addition to the Barr technique, making possible large-scale diagnostic examinations for detecting individuals with the anomalous

XYY chromosome which is responsible for a serious illness.

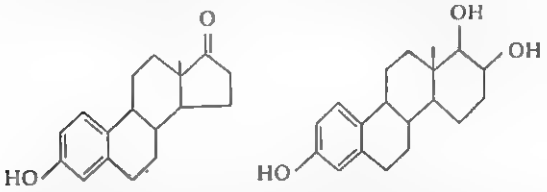
The luminescent Y chromosome is also detectable in other cell types, i.e. in blood lymphocytes, and in hair follicle cells.

Sometimes sex determination based only on the detection of the Y chromosome, can be misleading. This chromosome can have variable length which accounts for the variable length of the intensely luminescent part: in longer chromosomes it is markedly brighter. In the late seventies, researchers at John Hopkins University found a person with a very short Y chromosome that had no luminescent part at all.

Intensive luminescence of separate chromosomes or of their parts have been found only in human beings, in the gorilla and the chimpanzee, which thus links them evolutionally.

There is also another way that fluorescence analysis can be applied in the continuation of the human race. This method can assess the estrogen concentration, i.e. the female sex hormones, in human metabolic products (urine, blood plasma). The estrogen concentration differentiates ordinary plasma from that taken from pregnant women, and indicates whether pregnancy is following a normal course. The estrogen concentration in plasma increases in the course of pregnancy (usually 5-7 times). In toxicosis, this concentration increases

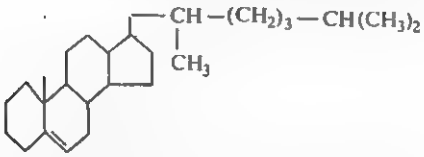
10-40 times. Fluorescence analysis is most useful in detecting two compounds of the estrogene group, i.e. estrone and estriol:



These structural formulas reveal an aromatic ring and phenol group in both estrogens. A specialist in organic luminescence would recognize them as natural luminescent compounds, although their emission would not be very intense nor would it be visible to the eye, because its peak is located in the UV region of the spectrum. For this reason, this natural luminescence is not employed in analysis; rather, the biological preparation is treated with sulphuric or phosphoric acid. As a result, a mixture of compounds is formed that luminesces with an intense blue colour. The rest is quite simple: the intensity of luminescence is measured and the estrogen concentration can thus be determined.

Estrogens are not the only biologically active compounds among the steroids. Cholesterol, corticosteroids, androgens and bile acids are also included. Among these, cholesterol is a compound that is most impor-

tant physiologically and can be easily detected by luminescence:



Cholesterol is virtually present in all the cells of the body; a lot of it is found in the blood plasma, lymph, adrenal glands, spinal cord, and liver. It is a precursor in the synthesis of sex hormones, bile acid, and corticosteroids. Cholesterol is also important as a regulator of cell permeability. Clinically, it is very important to know the cholesterol content in various tissues and organs. At high concentrations of the cholesterol in the blood, it can deposit on the blood vessel walls, initiating the development of atherosclerosis. In addition, impaired cholesterol metabolism results in the formation of crystalline cholesterol from bile (cholelithiasis).

For detecting cholesterol in blood by means of luminescence, it is first extracted by alcohol. The alcohol extractions are then evaporated and treated with a mixture of trichloroethane, acetic anhydride and sulphuric acid. The result is a complex mixture of compounds whose nature is not yet completely clear; however, this does not

prevent the practical application of this technique. What is most important is that the mixture displays intensive luminescence in blue and red regions of the spectrum. Quantitative measurements of cholesterol are carried out with respect to the intensity of red emission.

Hopefully, the above examples have informed the reader of the practical value of fluorescence analysis in assessing basic physiological functions of the body. However, modern medicine is not to be reduced to the laboratory observance of these processes, it far surpasses this limited task.

Control of Drug Intake and Transformation

Today physicians have at their disposal a huge variety of drugs. These drugs can influence physiological processes in many different ways. Every year thousands of new and highly active drugs are introduced into clinical practice. Many of these drugs are naturally luminescent (sometimes being very bright) or become so after simple chemical treatment. Thus, fluorescence analysis can be successfully applied for control of drug intake and for tracing its further distribution in various tissues.

The first and most characteristic drug of this type is quinine, which has been known since ancient times and is probably the first

that was known to be luminescent. The bright blue luminescence of quinine in sulphuric acid solution is indeed so intense that it is visible in concentrations as low as 10^{-9} g/ml. Since the design of the first fluorometer, i.e. a device used to measure the luminescence intensity of various objects, quinine has been used as a standard index. The procedure is extremely simple. The luminescent objects under investigation are placed next to the standard, and their emission intensities are visually compared (Fig. 4)*. The apparatus employed is equipped with a mercury vapour lamp, light filter, a sample cuvet, and mirror.

Of course, the possible applications of such an apparatus are limited. Today, the modern photoelectric fluorometer is used everywhere. The latter is able to detect even weak, invisible to the eye emissions, and at the same time to measure the intensity. The most widely used types of spectrofluorometers are listed and described in Table.

This table requires some comments. The excitation light coming from the source (usually from a xenon lamp) is transmitted through monochromator or interference filter before reaching the cuvet with the substance under investigation. This is necessary for isolating a specific spectral region

* This figure is taken from the book by S. Udenfriend, *Fluorescence Assay in Biology and Medicine*, New York-London, Acad. Press, 1962.

including absorption peak of the substance in the cuvet. The principal component of a monochromator is a diffraction grating

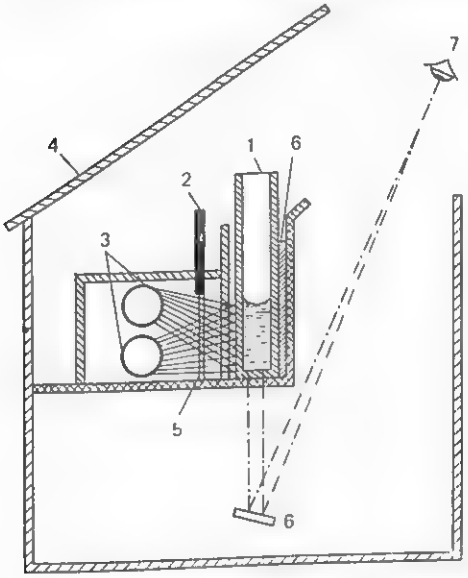


Fig. 4. The design of a visual fluorometer:
1—sample cuvet; *2*—light-restricting panel; *3*—mercury vapour lamps; *4*—lid; *5*—black glass filter; *6*—mirror; *7*—the observer's eye

providing a high degree of light monochromatism (about 0.1 nm). Interference filters, as compared to monochromators, have advantages as well as disadvantages. These

TABLE. Characteristics of Modern Types of Spectrofluorometers

Fluorometer	Region of measurement, nm	Scheme of cuvet illumination **
Neva	200-800	a
Jobin-Yvon-3	200-700	a
Aminco-SPF-125	200-800	a
Aminco-SPF-500	200-800	b
Aminco-SPF-1000	200-1000	b
Hitachi-Perkin-Elmer MPF-44a	200-700	a, b
Perkin-Elmer-1000	280-750*	
Jasco FP-550	220-850	
Kontron SPM-22	200-800	

* Interference filter (monochromator in all the other types).

** a, b–the scheme of beams in the cuvet compartment (according to Fig. 5).

filters have a 7-15 nm transmission band, and can transmit a flux of excitation light that is few times greater than that transmitted by a monochromator with the same band. However, the degree of light monochromatism is lower in the interference filters.

In all the fluorometers listed in the Table the angle between the excitation light and fluorescent emission is 90° (Fig. 5). In some cases, the excitation light can be focused in the centre of the cuvet (see Fig. 5*a*).

The fluorometric assay was for the first time applied by English scientists during World War II for determining quinine and medical agents with similar action, which

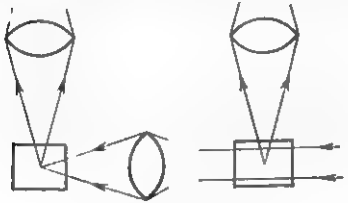
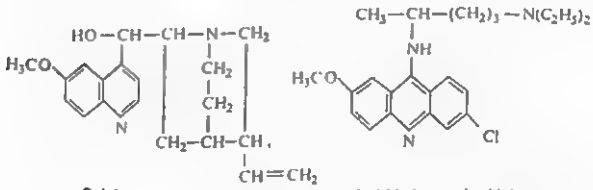
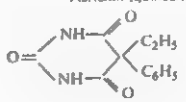


Fig. 5. The patterns of cuvet illumination in fluorescence measurements:
a—exciting light is focused in the centre of the cuvet; *b*—exciting light is incident on the cuvet as a parallel beam

were administered for malaria. The detection of quinine luminescence in urine was used as the criterion for determining the dosage of antimalarial drugs





The detection of quinine luminescence is the basis of a simple and original method of determining gastric acidity. When developing this method, scientists made use of the fact that quinine can be easily produced from cinchona tar or bark by treating it with an acid. For such an analysis, the patient ingests a certain amount of cinchona tar. The hydrochloric acid contained in gastric juice displaces free quinine from the tar, and this quinine is excreted with the urine. Measuring the intensity of quinine luminescence, one can determine its amount and therefore calculate gastric acidity.

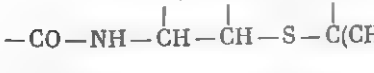
Luminescent antimalarial drugs can be derived not only from natural raw materials; they can also be synthesized. In the post-war years, effective synthetic drugs were developed, one of them being quinacrine hydrochloride (acrichin). It differs from quinine in that its molecule has acridinic cycle, and the structural differences between quinine and quinacrine hydrochloride determine the different luminescent properties of the two substances. Quinine is not luminescent in alkaline medium, while quinacrine hydrochloride produces bright green glow in this medium.

Antimalarial drugs are not the only drugs that have the property of luminescence. In fact, many analgesics, cardiovascular, and neurological drugs such as aminazine (chlorpromazine) and reserpine, are naturally lu-

minescent and can thus be traced within various organs of the body. Most barbiturates display a marked luminescence in alkaline medium, the colour varying from violet to amber. The well-known luminal (phenobarbital) has an intense blue glow at pH of 13.

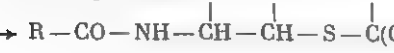
Several of the vitamins (A, B_2, and others) are also luminescent. Vitamin A (retinol), which is administered when there are various defects of the corneal epithelium, or when peptic diseases or eye disturbances are present, has an intense green emission in alcohol solutions. The emission is due to the presence of five conjugated double bonds. It is so bright that vitamin A can be easily detected in amounts less than 10^{-6} g in excretion products.

There is, however, a problem when dealing with drugs that are not naturally luminescent. One solution is to modify the molecular structure by chemical reactions. This can be illustrated by the procedure for the luminescence detection of antibiotics. The structure of the antibiotic can be represented as follows:

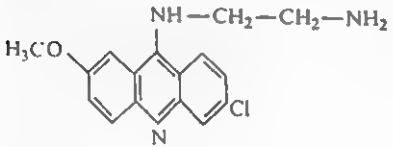


To make penicillin molecules luminescent, they must be reacted with aliphatic

amines:



For the reaction with penicillin, an amine with a luminophore part is taken that can make the amine and its derivatives luminescent. For this purpose 2-methoxy-6-chloro-9(β-aminoethyl)-aminoacridine is often used:



The product of the reaction between penicillin and this amine contains the luminophore acridin group, and thus possesses a bright green emission. The simplicity of the described procedure and high accuracy of measurements make it possible to use fluorescence method for the detection of penicillin in blood and urea.

Thus, this is a clear demonstration of the medical value of fluorescence analysis in the control of drug intake and excretion. Let us now consider its application in diagnostic procedures.

Diagnostics

Routine luminescence diagnostic procedures. A discussion about the versatility of luminescence techniques applied in medical diagnostics, should first be introduced by outlining the specific methods of detecting infectious agents, i.e. bacteria, viruses, fungi, and other organisms. The principal problem that arises when we deal with these organisms is their lack of natural luminescence. However, a small number of exceptions do possess this property, and they will be discussed at length later in the section entitled "Luminescence and Food Quality Control". Here, one illustrative example is quite sufficient, that of a luminescent fungus responsible for the onset of microsporia, a fungal pathology affecting hair. These fungi, in fact, do not luminesce, but their enzymatic action on the affected hair yields secondary products that luminesce green. Back in the thirties this phenomenon was the basis for large-scale examinations of children. The examinations were performed in a dark room by means of a portable UV lamp. This method proved to be effective in preventing microsporia in children.

In order to detect a vast majority of pathogenic microorganisms, they have to be stained with a luminophore. Direct staining of human or animal tissue preparation

or smears, as well as of soil samples and water washings of the objects to be analysed, makes it possible to detect microorganisms in them and perform quantitative determinations. Moreover, luminophore staining allows one to differentiate between viable microbes and those that are already dead or irreversibly damaged. The fact is that dead microorganisms bind a larger amount of luminophore and thus acquire a brighter emission than live microorganisms. The diagnosis of diphtheria, leprosy, malaria, and tuberculosis is based on different degree of staining of microorganisms by luminophores.

Auramine and the above-mentioned acridine orange are luminophore stains that were used for this purpose more frequently than other stains. The past tense is used here because a newly developed immunofluorescence method has been recently introduced in medical practice to detect pathogenic microorganisms. This technique is highly sensitive and specific, and has met the various medical requirements. A separate section of this chapter will be devoted entirely to the description of this method.

Fluorescence microscopy can detect young colonies of microorganisms placed on membrane filter that cannot be observed by the naked eye. The application of acridine orange makes possible the detection of spe-

cific phagolysis* in small colonies of young dysenteric bacteria (Fig. 6).

The reader might have noticed that young colonies of bacteria were considered above.

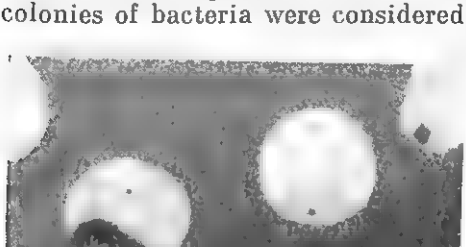


Fig. 6. Phagolysis in young populations of dysenteric bacteria stained with acridine orange (80-fold magnification)

What does the word "young" mean as applied to bacteria? Young bacteria are those whose age is no more than a few hours, the threshold in this particular case being 7 hours. Therefore, the control of these bacteria must be performed quickly. The ancient Roman adage can be repeated here: "He who gives at once gives twice as much." Fluorescence analysis, which can be performed in just a few minutes, meets these requirements. The use of membrane filters as substrates has made the identification of

* Phagolysis is the destruction of microorganisms under the action of bacteriophage (a virus that lyses bacteria).

bacteria even more simple. A solution of bacteriophage and acridine orange can be more easily applied to the bacterial colony from the opposite side of the filter.

Luminophore staining is also of great help in elucidating the structure of bacterial cells. The data of fluorescence microscopy, together with cytological data obtained by other techniques allowed one to distinguish between the nuclear and cytoplasm within the bacterial cell.

The reader is probably familiar with the widely quoted statistical figures of cancer mortality, which put cancer in second place after cardiovascular mortality. This is the situation in the USSR, as well as in the majority of developed countries in Western Europe and North America. Today, everyone uses the word "cancer", but does everyone correctly understand the meaning of the term? What do specialists mean by the word "cancer"? It is known that the numerous cells that make up the human body are constantly divided and increase in the number. A typical example is the process of wound healing when the number of neighbouring undamaged cells increase and cover the wound. In a healthy organism there is a stable equilibrium between dividing and dying cells. Physicians usually speak of a specific mechanism regulating the processes of cell division and tissue growth. This mechanism, however, does not always func-

tion perfectly and may not adequately control cell division process. The equilibrium may then be upset and cell devision proceeds uncontrollably. It is this disturbed equilibrium that leads to the development of malignant tumours that are known as cancer*.

The highest incidence of cancer has been recorded in New York City, whereas the lowest incidence was found in Sri Lanka. Why this is so, nobody as yet knows for certain.

Sometimes people that are not well acquainted with medicine and its history make the conclusion that cancer is a new disease that was formerly unknown to man. This is far from the truth. Cancer is referred to even in the ancient manuscripts of India and Egypt. The writings of Hippocratus dating back to the fourth century B.C. give an accurate and detailed description of various malignancies. One of the reasons why cancer was rather "unpopular" in the past is because its development is relatively slow. In comparison with such epidemic and devastating diseases as the pox, plague, and cholera, cancer certainly looked "modest".

But advances in microbiology and epidemiology have eliminated the danger of

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* More precisely, cancer is a process of cell multiplication, involving one type or clone of cells.

formerly inconquerable epidemics. In the Soviet Union, there have been no cholera epidemics since 1926, and pox epidemics were eradicated a decade later. Today, typhoid fever, cholera, and dysentery are under control in the USSR. Unfortunately, this is not the case everywhere in the world. The only infectious disease that has been reduced to single cases per year is "black death", i.e. plague.

After the impressive progress made in this century in controlling epidemics, the time for the close scrutiny of malignant diseases has arrived because they are now one of the leading causes of mortality among the existing pathological conditions. Because of its leading role in mortality statistics, uninformed people may believe that cancer is incurable. However, cancer can be cured, and the earlier a diagnosis is made, the quicker and more effective is the treatment. To completely control cancer, scientists must find out everything concerning this condition. Unfortunately, this has not yet been realized. As yet, even the cause for the uncontrollable cell division that leads to cancer is unknown. What is the nature of malignant tumours?

The theory about the infectious nature of cancer, which was once rather popular, today has few advocates. It was strongly contradicted by an experiment, where an oncologist grafted a tumour, resected from

his patient, onto his own body. The transplant did not result in cancer development. However, some epidemiologists, in particular Academician O.V. Baroyan, do not deny the possibility that malignancies, as well as cardiovascular and mental diseases, are, in some way, infectious. Thus, the transplant experiment, which did not result in the development of cancer is explained by scientists in the following way: the mechanisms stimulating the appearance of cancerous cells are genetic, but those that are involved in controlling the spread of malignancy are immune.

In the last 5-10 years there has appeared and been successfully developed a hypothesis proposed by A. Knoodson. According to this hypothesis, tumours originate from one single cell. An important part of the hypothesis is the concept of mutation, which is a hereditary change in the nucleotide sequences resulting from a change in the primary DNA structure. Hereditary forms of cancerous tumours are explained by the fact that one of the mutations required for tumour development is contained in all the cells of the given organism, since it is inherited from the parents. As a result of the first mutation, the cells become susceptible to the second "blow", which initiates malignant tumours.

The appearance of acquired, non-hereditary tumours requires at least two mutations

within one and the same cell. The probability of this occurring is very low, and therefore this type of tumour is usually found in elderly patients and is manifested by single cases.

The more cancer is being studied, the more evident it becomes that it is not only a medical problem; more importantly is it a biological problem.

All in all, the annual cancer mortality reaches several millions, therefore, the necessity of developing efficient methods of early diagnosis is obvious. Today, no scientist doubts that cancer never occurs in normal healthy tissues. Malignancy is always preceded by a long period of pathological changes in the organism. These changes, which sometimes take years, are termed "pre-tumorous", or "pre-cancerous" by medical researchers.

The diagnosis of malignancies that can be observed is not difficult. Sometimes the patient himself notices newly-formed palpable tumours, ulcers, areas of skin thickening, or painless swellings, which require that he consult a doctor. There are also some well-known cases when cancer of internal organs can be detected early. For example, gastric cancer is manifested by postprandial discomfort. In this case a visit to an oncologist must not be delayed.

Another symptom of gastric cancer is a vascular formation on the anterior abdomi-

nal wall which is called *caput medusae* (Medusa head). This unusual name originates from the Greek myths. The reader probably remembers the three mythical Gorgon sisters of hideous appearance and evil disposition. Medusa, one of these three, had wriggling snakes on her head instead of hair, and also, with a glance, she could turn any living creature into stone. Perseus, like many other mythical heroes, set out to kill Medusa. The main problem was, however, to approach Medusa and accomplish this task without looking at her. The wise Perseus took a shield that was polished like a mirror, and, guided by Medusa's reflection in the shield, he severed her head. This head, wrapped up in goat's skin, was later used to free the beautiful Andromeda, who, according to the myth, was chained to a cliff and tormented by a sea monster. Perseus, standing on the seashore, unwrapped Medusa's head and showed it to the monster, which immediately turned into stone.

One may wonder about the relationship of this ancient myth to the diagnosis of cancer. It is simply that an oncologist "freezes" at the sight of the *caput medusae*, because it is a manifestation of such an advanced stage of the disease when the patient can no longer be helped. However, other organs affected by cancer, do not have such obvious external symptoms even at

very advanced stages. How then can the other forms of cancer be diagnosed?

The reader may have guessed already that, here again, fluorescence analysis can be used. This method was first applied to cancer diagnosis a few decades ago. The procedure was based on the simple observation that the luminescence of the affected tissues, blood and urine in patients with malignancies is markedly brighter than in healthy people. Some researchers came to the conclusion that the colour and intensity of skin luminescence can be used for an early diagnosis of precancerous conditions. The following observations have been made: necrotic areas of malignant tumours have a red or orange emission; that of skeletal or cartilage malignancies is a bright blue, while benign tumours, are not luminescent at all. Luminescence analysis can also assess treatment efficacy: when the emission disappears, the cancer can be considered completely removed.

Medical investigators have known for a long time that malignant tumours can accumulate a variety of luminescent substances, and this property has been made use of in diagnostics. How is this done? The patient who is suspected to be suffering from cancer is injected with a luminescent dye. In healthy subjects, the dye (fluorescein) will be completely removed within 50-70 hours, but in cancer patients, it might still be de-

tectable after as long as 2000 hours.

This procedure was introduced more than 20 years ago to facilitate the surgical resection of lung cancer. It was done in the following manner. The patients were given a large (up to 1 g), but harmless, dose of fluorescein 3 to 4 hours before the operation. On the operating table, under UV illumination, the tumour of the patient was clearly outlined: malignant tissues luminesced with a yellow-brown colour, while the healthy areas were greyish-blue.

Lung surgery is not the only example of this type. Fluorescein solution can reliably outline cerebral malignant tumours, thus also providing a control for its surgical removal. Pre-operative rivanol injection, which produces a bright luminescence, is also a reliable means for controlling laryngeal tumour resection: a difference in the intensity of the luminescence colour distinguishes between the malignant and normal tissues. Some specialists recommend the injection of up to one gramme of hematoporphyrin (a harmless and intensely luminescent substance) for the detection of intestinal tumours. Its red emission is so intense that it can be observed *in situ* through the intestinal walls.

The ability of malignant tumours to accumulate luminophore substances, which makes them visible under UV light, has already been discussed above. This pheno-

menon can be related to macroeffects, which consist of microeffects. The microeffect is based on the ability of cancerous cells to accumulate a greater amount of luminophores as compared to normal cells; this makes possible the differentiation between stained malignant and normal cells. Obviously, a fluorescent microscope is required for these studies.

M. N. Meysel, a prominent Soviet scientist, conducted a series of pioneering studies in the field of fluorescence cytodiagnosis of cancer. Twenty five years ago he and his coworkers studied a large series of cases which included malignancies of the larynx, tonsils, oesophagus, prostate, and urinary bladder. In all these conditions malignant cells were easily detectable by fluorescence microscopy in smear, punctate, and scrape specimens.

The analysis was quite simple. The scraping was carefully made by a very small scraper from the surface of the organ suspected for malignancy, and then is immediately placed on a microscopic slide into a solution of luminescent dye. After a series of trials, the best dyes were found to be very dilute aqueous solutions of acridine orange or coriphosphine (1 : 2500). The procedure of staining took only a few minutes and the specimen was ready for examination under a fluorescent microscope.

What is then the difference between the

luminescent properties of malignant and normal cells? An illustrative example is the cells of squamous epithelium of mucosal membrane stained with acridine orange.

Normal cells when stained have green colour varying in shade and intensity throughout the cellular structure: protoplasm luminesces with a dull green colour, whereas the cell nuclei are bright green. Acridine orange is bound to the protoplasms of young and old cells in a different way and thus imparts a different colour to them, which allows for the detection of early stages of cell cornification. The dye can also reveal accumulation of metabolites in cells, as well as quantitative changes in RNA concentration. The RNA and its derivatives are easily bound with luminophores into a complex which luminesces with a bright red or orange colour. By observing the colour variations one can study changes in the cellular structure that are caused by inflammatory or other pathogenic processes.

We have already noted that tumour cells adsorb luminophores more actively than normal cells. As a result, their emission is more intense, and the colour of their emission is shifted to the red region of the spectrum.

Tumour cells have unusually large, balloon-like nuclei, which are often irregularly shaped and have large pink or even red-

coloured nucleoli. The protoplasm within such cells luminesces with orange or light pink colour. There are also various bodies present in it with a bright red emission.

Fluorescence cytodiagnosis has many advantages over routine cytodiagnosis in that it is simpler, more evident, and less time-consuming. It is not surprising that it has been applied to large-scale cancer screening of the population. It has also been essential for investigating malignant tumours of the female genitals. Vaginal and uterine malignancies are actually very high on the list of serious diseases affecting women. The American Cancer Society has stressed the decisive role of early diagnosis. Data published in 1957 reveal the following success rates in the treatment of cervical cancer: stage I (initial)—70 per cent, stage II—49 per cent, stage III—31 per cent, and stage IV—8 per cent. In recent years, the results obtained in most Soviet oncological centres are much more better. Obstetrics and gynaecology were among those fields of medicine that first applied fluorescence cytodiagnosis. The techniques described above are well suited to specific gynaecological problems. Cancerous cells stained with luminophores display rather large red nucleoli within their nuclei.

In the last decade, ophthalmic cancer has been intensively investigated by observing the circulation of fluorescein in the blood

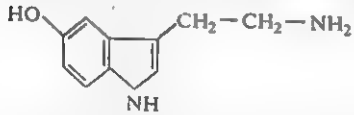
vessels of the retina. This method, which was called fluorescence angiography, is based on serial photorecordings of the fundus of the eye during the circulation of the fluorescent dye in the blood vessels. Such studies have been conducted at the P. V. Filatov Institute of Ophthalmology and Tissue Therapy in Odessa. The instrument used for photographing is called "Retinophot" and is a product of Karl Zeiss Company (GDR).

The fluorescein solution, introduced into the blood circulation fluoresces intensely when illuminated by blue-violet light. The lens of the camera is equipped with an amber-green light filter. It absorbs almost all of the reflected blue-violet light, at the same time transmitting the green rays that are emitted by the fluorescing blood vessels: they are thus clearly delineated against a dark background.

Fluorescence angiography is used not only in the diagnosis of ophthalmic malignancies, but also for recognizing and controlling the treatment of diseases of anterior of the eye. For this purpose a special illumination device was designed at the same medical centre in Odessa. The apparatus contains an electric incandescent lamp and a gas-discharge lamp. The latter is capped with a blue-violet light filter. Another light filter which is amber-green in colour is fitted on the lens. The lens is connected

to the camera body by a draw tube.

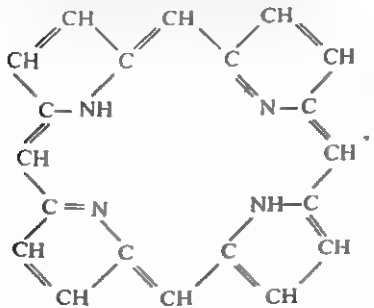
Somewhat apart from the fluorescence methods of tumour diagnosis, which is based mostly on the affinity of malignant cells to luminophore dyes, there are diagnostic techniques for another type of cancer, the so-called malignant carcinoid. This dangerous tumour is located in the intestine, from where it spreads through the blood circulation into various visceral systems, reaching even the brain. It differs from ordinary cancer by the properties of the affected cells, and also by its clinical manifestations. A most characteristic feature of carcinoid underlying its luminescence detection procedure is the production of serotonin, an amine with high physiological activity and bright luminescence. The structural formula of serotonin is



Anyone acquainted with organic luminescence will not fail to note that the luminescence of the molecule is due to the presence of 5-oxyindole ring. All compounds containing such rings have a bright amber-green luminescence in acidic medium. Thus, if carcinoid is suspected, the diagnosis can be verified by a blood analysis for serotonin concentration. After the precipi-

tation of blood proteins and addition of acid, the resulting emission is observed. If it is markedly amber-green in colour, then the serotonin concentration must be very high, as well as the probability of carcinoid.

The diagnosis of carcinoid is an example of the almost seemingly conscious striving of nature to meet the requirements of fluorescence analysis: the human organism, in response to the destructive influence of tumour, starts to produce serotonin, a brightly luminescent, and, therefore, easily detectable compound. This is not, however, the only luminescent substance produced. Physicians frequently make reliable diagnoses on the basis of luminescent substances found in secretions. Porphyrins, which were discussed above, are most important in this respect. They have a common structural element, i.e. the porphyrin ring, which is responsible for their characteristically bright red emission:



Porphyrins are indispensable participants in the vital activity of the human organism. They are a component part of hemoglobin molecules, as well as of erythrocytes. The latter produce only a very weak red luminescence in healthy subjects; however, in several pathological states, the intensity increases greatly because porphyrin content increases 3-40 times above normal level. What diseases induce such a change in porphyrin content?

First of all, a deficiency of iron in the diet; second, an increase in erythropoiesis (a term derived from the Greek *erytros*—red, and *poiein*—to form, i.e. increased hemopoiesis), which can be seen in some cases of anemia; and third, lead poisoning, as occurs in industrial workers. The last example warrants a more detailed discussion, because the condition is encountered more frequently than the others. If fluorescence analysis reveals high porphyrin content in the red blood cells of industrial workers, one can be unequivocally certain that lead poisoning has occurred.

In practice, urine tests are more convenient for the fluorescence diagnosis of lead poisoning than blood tests. In the early stages of lead poisoning a 2-10-fold increase in urine porphyrin level is observed. This increase can be detected by a very effective and simple diagnostic procedure. It is as follows: a certain volume of urine sample

is treated with ether; ether extracts are acidified with a 5% hydrochloric acid solution, and are then examined by means of a visual fluorometer. When the porphyrin concentration does not exceed normal levels (i.e. 0.075 mg/l), the ether layer luminesces with light-blue or greenish colour. In cases of lead poisoning, the colour turns to pink, and its intensity indicates to what stage the condition has progressed. Sometimes, in the most severe cases, even a red glow has been observed.

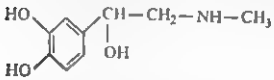
The instruments used for the fluorescence analysis of urine samples are so simple that they can be constructed right at those enterprises where lead or its derivatives are employed in the industrial process. The porphyrin content can be determined within a few minutes: a laboratory assistant can perform at least 50 to 60 tests a day. Thus, the periodic screening of workers can be carried out without much effort.

It should be noted, however, that lead poisoning is not the only condition characterized by an increased content of porphyrins in the urine. A 3- to 4-fold increase is seen in some hepatic disorders, in poliomyelitis, and also in lymphogranulomatosis, or Hodgkin's disease (a condition manifested by tumorous growths of the lymphatic nodes and affecting the spleen, liver, and bone marrow).

Other cases are known in medicine when

the situation is the reverse and the urine porphyrin level decreases 4-10 times below normal levels. This is often found in uremia, a disease manifested by the excessive accumulation of protein metabolites (urea, uric acid, and others) in the blood. The cause may be impaired renal function, or urologic diseases. Fluorescence analysis can also detect this decreased porphyrin content and can assist in diagnosis of the disease.

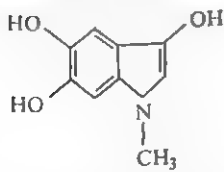
The fact that the diseased organism excretes brightly luminescent compounds finds its application in the diagnosis of hypertension. In fact, this particular type of a cardiovascular condition can develop in a variety of situations, one of the causes is the well-known arterial hypertension; the other is phaeochromocytoma, i.e. a tumour of the adrenal medulla, or of the ganglions of the sympathetic vegetative nervous system. This type of tumour is peculiar in that it produces luminescent catecholamines, which are biologically active compounds. The most important among them are adrenalin and noradrenalin:



In normal subjects or in those suffering from hypertension the daily urine catecholamine level usually is about 32 μg, as demonstrated by fluorescence analysis,

whereas in patients with phaeochromocytoma it may be as high as 200 μg. The fluorescence testing of urine for catecholamine concentration appeared to provide an easy diagnostic solution to the problem. However, in practice some serious difficulties arose.

Although adrenalin and noradrenalin are both luminescent, their luminescence is not specific and intense enough, therefore they cannot be distinguished from a multitude of other similar compounds. Thus, specialists in fluorescence analysis had to turn for help to the chemists who developed simple and highly specific methods for transforming catecholamines into brightly luminescing compounds. One of these methods, "trioxyindole" method, consists in oxidation of catecholamines by iodine with subsequent treatment with an alkaline solution of a salt of ascorbic acid. This reaction transforms adrenalin into trioxyindole:



This compound luminesces with a greenish-yellow colour. The method was named after the compound.

Noradrenalin reacts in the same way and is transformed into a product with a similar chemical structure and a similarly coloured emission. By measuring the intensity of the luminescence of these products, the initial catecholamine concentration in the urine sample can be easily determined.

The determination of urine and blood catecholamine levels is important not only for making a correct diagnosis of hypertension. It is known that adrenalin and noradrenalin of the adrenal medulla are among the most reactive substances, and thus play a vital role in the important physiological processes of the human organism. Catecholamines were first identified due to their pharmacological effect. Highly sensitive techniques for their quantitative determination, which do not require the use of any sophisticated equipment, were developed that were based on their biological activity.

However, today the biological analysis of catecholamines does not meet the demands of specialists. This is not without reason: first of all, it cannot be applied to biochemical studies, and, in addition, more and more new physiologically active agents are being prepared that can interfere with the pharmacological action of catecholamines. Here, fluorescence methods came to the rescue for the determination of adrenalin and its derivatives.

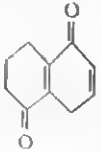
Several years ago, investigators from abroad made a suggestion that an intermediate compound, aminochrome, formed during an oxidation of adrenalin into trioxyindole, had a hallucinatory effect. Fluorescence analysis of blood samples taken from patients suffering from schizophrenia showed a markedly higher aminochrome level as compared to normal subjects. It is possible that this observation will lead to the development of new diagnostic procedures for early stages of this frightening illness.

We have thus discussed the quantitative determination of weakly luminescing compounds like catecholamines, which require preliminary chemical treatment. This type of treatment is also required for the identification of sugar and vitamin B_1 which are not naturally luminescent substances.

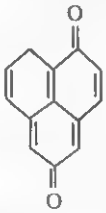
Everyone is aware of the important role of carbohydrates in the functioning of the human organism. Thus, it is not surprising that medical specialists are interested in techniques for determining sugar content in different organs and tissues. By way of illustration let us consider sugar determination in blood. The content of carbohydrates (primarily of glucose and glycogen) in the blood is regulated by the nervous system, endocrine glands, liver, and muscles. In diabetes, the content of carbohydrates in the blood usually increases 2-5 times.

It becomes obvious that the determination of sugar in the blood has a diagnostic value.

Fluorescence method for detecting glucose and its derivatives in blood was developed in 1959 by Japanese investigators. Its sensitivity appeared to be higher than that of other techniques. This method is based on the reaction between glucose and

5-oxy-1-tetralone  in the presense

of strong acids. The products of the reaction are luminescent. Indeed, the main product

is benzonaphthenedion  which

has a bright amber-green emission. By measuring the luminescence intensity it is possible to determine glucose content in the blood sample. The unusual sensitiviy of the test has been mentioned already, but one of its advantages is the minute amount of the blood sample needed to accomplish the procedure: it might be as

low as 0.001 ml. This fact alone speaks for itself.

An analogous procedure has been used for identifying vitamin B_1 (thiamine). This vitamin belongs to the group of water-soluble vitamins, and is very important as an enzymatic mediator in many physiological processes. A deficiency of vitamin B_1 results in the disease beriberi. This is very dangerous disease which affects the peripheral nervous system, and is accompanied by limb muscle atrophy and oedema. The search for the cure for this disease is an interesting story, which led to the actual discovery of vitamin B_1.

In the last century beriberi was most commonly found among the native populations of South America, East Asia, and of the Pacific islands. The people of these regions, who subsisted mainly on rice, died by the thousands of this insidious disease. Beriberi was first manifested by paralysis as well as numbness in the lower limbs; then, the cardiac and pulmonary systems gradually deteriorated. Death was sudden and agonizing, caused by suffocation. In 1886, the Dutch government was so disturbed and alarmed by the incidence of this disease in its colonies that it dispatched a young physician to these areas whose aim was to find the cause of beriberi. His name was Christian Eijkman. The studies were carried out for a long period of time and

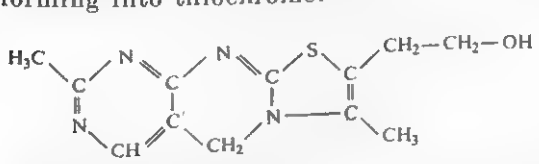
seemed to come to a dead end, but, as frequently happens, a clue arose from a chance observation.

The young investigator noticed the strange behaviour of chickens belonging to the laboratory where he worked. These chickens suffered from paralysis and a variety of symptoms very much resembled those seen in beriberi. He at once started to study this condition with great zeal, but, disappointingly, his work was interrupted by the inexplicable recovery of the chickens. There was nothing more to study as no new cases appeared. But reasonably suspecting that the disease was caused by the feed given to the chickens, Eijkman checked into this. It turned out that just before the onset of the disease the chickens were fed on polished (white) rice, i.e. with the husks removed. This rice was intended for the local military hospital. But when the head cook was replaced by another, Eijkman himself humorously remarked when he was awarded the 1929 Nobel Prize in physiology and medicine, the new boss did not allow that any more of "the army's rice be supplied to civilian chickens". It was at this moment that the condition of the chickens began to improve. After this observation the chickens were divided into two groups: one group was given polished rice, and the other, rice that had not been husked, i.e. ordinary rice. Predictably, all chickens of

the first group died within two or three weeks, while the others remained perfectly healthy.

The next step in this investigation was to extract from rice the substance that would prevent beriberi. Years of careful and detailed studies on the physiological action of rice husk extract revealed that it contains a nutritional substance indispensable for the normal functioning of the human organism. This substance extracted from rice husks was given the name "vitamin B_1" (from the Latin *vita*—life and *amin*—the chemical term for a functional group in nitrogen compounds). The name itself emphasizes the vital importance of the substance.

Thus, physicians required an easy and reliable method of identifying vitamin B_1 for the diagnosis and prevention of beriberi and other dangerous conditions. Such a method was discovered. Thiamine, which is not naturally luminescent, becomes so when oxidized in alkaline medium, transforming into thiochrome:



It has a blue luminescence which can be easily measured. This technique can be used to detect vitamin B_1 and determine its

content in blood, urine, and food without difficulty.

Immunofluorescence technique. The development of this new technique provided qualitatively new opportunities for the application of luminescence in medicine, and particularly, in the field of medical diagnosis. The method was first developed by A. Coons. In the forties this American investigator accomplished the chemical binding of a dye (fluorochrome) to antibodies, the functional elements of specific serum proteins (immunoglobulins). These labeled immunoglobulins were named "fluorescent antibodies" which explains the other name of the method—fluorescent antibody technique.

In the Soviet Union research into this field was begun in the 1950s. What is the general idea of this method? The essence of the new technique by A. Coons is the luminescence of the well-known antigen-antibody reaction that occurs when antigens bind to corresponding specific antibodies stained with luminophore substances.

This technique is widely applicable in diagnosis. For example, it is successfully used for the identification of different corpuscular antigens, such as bacteria, viruses and rickettsiae in pure and mixed cultures, and also in print preparations, and organ and tissue sections. Later on, we will discuss the application of the immunofluo-

rescence techniques for the identification of causal agents of various diseases. Now let us discuss the existing versatile modifications of the method. This is even more necessary in that it enables one to understand more about the nature of the immunofluorescence phenomenon.

The direct and indirect methods are the ones that are applied more often than oth-

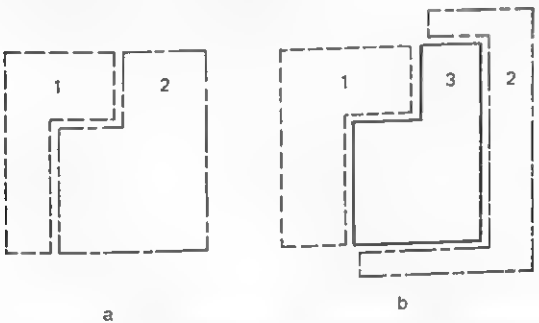


Fig. 7. Two methods of fluorescent antibody technique:
a—direct method; *b*—indirect method; *1*—antigen; *2*—luminescent serum; *3*—antibody

ers. Figure 7 graphically illustrates the difference between these two methods. The main step in the direct method is the treatment of the specimen with a corresponding luminescent serum, which is obtained by binding antibody to a luminescent stain. The following steps are virtually the same as those in routine luminophore staining of

biological specimens. The excess amount of non-bound luminescent serum is decanted, after which the specimen is washed in order to remove the serum residue; the specimen is then placed under a fluorescent microscope.

The indirect method mainly differs from the direct one in that it is a two-step procedure. The first step is the binding of antigen with non-stained serum resulting in the formation of a non-luminescent antigen-antibody complex. Next, the complex is treated with the luminescent serum to the particular antibody (with antiglobulin). A specific circumstance must not be overlooked here: in order to obtain antiglobulin, the host animal from which the specific serum (i.e. the "first" antibody) is to be taken, must, prior to that, be made immune by a serum (globulin) injection. Moreover, the host must be of a different species. For example, to obtain antibodies to human globulin, human serum must be injected into the rabbit.

What then are the advantages of the direct method? First of all, it is a very simple procedure that practically reduces to zero the probability of diagnostic errors. Therefore, it has been widely applied to identify pathogenic microorganisms. But perfection is very rare indeed. The direct method also has its disadvantages, the most discouraging of them being the necessity of obtain-

ing luminescent serum for every antigen type. This immediately reveals the advantage of the indirect method which makes use of only a small variety of sera (mostly those to rabbit and horse globulins). However, this method is also not completely ideal, because it requires the participation of supplementary protein reagents in the reaction, which increases the probability of diagnostic errors.

Depending on the research problem, the investigator chooses one of the two methods. The best results can be obtained when both techniques are used, because, in this way, the antigenic properties of microorganisms and their specific features will be revealed more completely.

Let us now consider the practical application of the immunofluorescence method in medical diagnosis, beginning with the identification of pathogenic bacteria. Bacterial antigens can be very conveniently detected in surface structures of microbial cell such as capsules, cell walls, as well as in other organoids. This is very easily done. The biological material under investigation is routinely prepared. The smears of the material are applied on a carefully degreased microscopic slide, nearer to its edges. After being air-dried, the preparations undergo fixation by immersion for 15-30 min in ethyl alcohol or acetone solution. They should not be stored, but should be treated immedi-

ately with a luminescent serum. At this stage, the bacteria can be identified by both direct and indirect procedures. After the

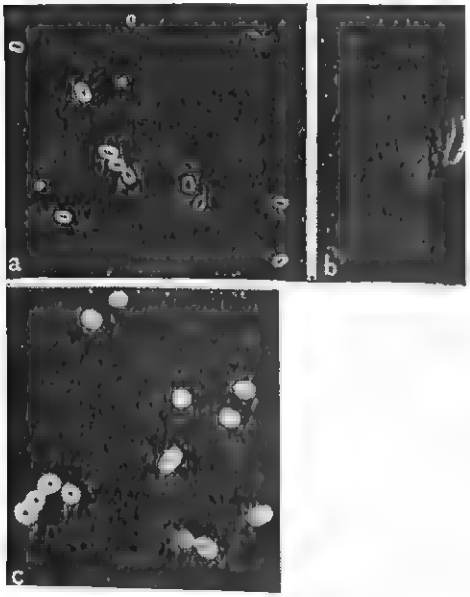


Fig. 8. Bacteria treated with luminescent sera: *a*—dysenteric bacteria; *b*—comma bacilli; *c*—Brucella

excess amount of serum is decanted, the specimen is ready to be examined under a fluorescent microscope.

What does one see? If bacteria are actually present in the specimen under investi-

gation, and the procedure has been accurately carried out, one will see a picture similar to that in Figs. 8 and 9.

After treatment with luminescent sera, bacteria emit a very bright luminescence,

Fig. 9. Capsule forms of anthracic bacteria as revealed by the immunofluorescence technique

which is however limited to the cell membrane, while the inner cell structures remain dark. This type of emission is termed specific, and differs from non-specific emission which is distributed throughout the cell due to the excess luminophore in sera used in the staining procedures.

Another very important advantage of fluorescent antibody technique is its extraordinary sensitivity. Compared with routine immunological techniques (for example, the agglutination reaction method), which are effective only in media where the bacterial concentration is several millions or even billions cells per millilitre, fluorescent antibody technique can detect bacteria

present in concentrations as low as 10^5 cells per millilitre.

The high sensitivity of the new technique compared with routine methods is demonstrated by its application in laboratory tests for the diagnosis of dysentery. This technique can detect the presence of *Shigella*, one of the pathogenic agents, in 90.6 per cent of patients, whereas the standard bacteriological procedures reveal only 65.6 per cent of patients.

The immunofluorescence technique for the diagnosis of typhoid fever has been found to be no less sensitive and efficient. Immunofluorescence blood testing in 96 patients with typhoid fever yielded positive results in 30 patients, whereas routine bacteriological methods yielded positive results in only 18 patients.

However, it is necessary to add here that such good results were obtained only after increasing the bacterial concentration in the specimens by placing them in culture media. For dysentery, this medium was a broth with chloramphenicol; and for typhoid fever, a broth with bile. Unfortunately, the sensitivity is markedly less if the specimen is not previously cultured.

Another indisputable advantage of the immunofluorescence technique is the developing capability to identify and differentiate a specific antigen to be tested in mixed antigen media. This is a promising prospect.

But again, if the concentration of the admixed bacteria is too high, the sensitivity of the technique decreases 10-100 times.

How can the decrease in sensitivity be dealt with? A number of ways have been suggested. The most interesting one among them appears to be a method that employs sera with differently coloured luminescence. For example, in 1969 it was suggested that to detect enteropathogenic (from the Greek *enteron*—intestine) rods, conjugates stained with fluorescein derivatives (green emission) and rhodamin derivatives (orange emission) should be used. This idea was further developed. In 1973 Soviet investigators prepared differently coloured luminescent antibodies to 14 serologic types of *E. coli* and to 2 types of dysenteric bacteria. The effectiveness of this modification of immunofluorescence technique in diagnostic practice can scarcely be exaggerated.

The diagnostic possibilities of fluorescent antibody technique are by no means reduced only to the diagnosis of dysentery and typhoid fever. This technique has also been introduced in many countries as a standardized diagnostic test for colienteritis, tonsillitis, gonorrhea, syphilis, and other types of infectious diseases. At present, the immunofluorescence technique can detect and differentiate almost any of the known pathogenic agents of bacterial infections. The enumeration of all these tests

is not necessary. To make a long story short, the author wishes only to present a brief outline of the promising new diagnostic procedure for syphilis.

The fluorescent antibody technique has been very effectively applied to the detection of antibodies to treponema in the serum and spinal fluid samples taken from syphilitic subjects. These antibodies are produced as a natural response to infection with *T. pallidum*. This method has advantages over standard serologic procedures for the diagnosis of syphilis in that it can demonstrate the presence of specific antibodies in serum not only during the incubation period, long before any clinical manifestations are evident, but even in seronegative syphilis, when all the other existing serological tests give only negative results.

Finally, the immunofluorescence techniques for the diagnosis of viral and rickettsial infections should be discussed. The techniques do not fundamentally differ from the standard procedures for detecting bacteria. The first step, as usual, is the preparation of the specimens to be investigated by taking smears on a glass slide. The source from which the sample is taken depends upon the type of pathogenic agent. Usually tests are performed on blood, sputum, spinal fluid, and, in cases when dermal eruption or rash is present, on the contents of vesicles and crusts.

The next step is the fixation of the specimen on the glass slide. The procedure is routine and guarantees that the smear cannot be washed off the glass slide, protects the investigator from being contaminated, and provides for closer contact between the luminescent serum and the intracellularly located viruses and rickettsia. The latter condition requires more detailed discussion.

The intracellular penetration of the luminescent serum molecules is indispensable for the formation of antigen-antibody luminescent complexes in cells contaminated by viruses or rickettsia. The main obstacle is that normal cells are impermeable to large luminophore-stained molecules like globulin. This obstacle is, however, overcome by fixation which makes the intact membrane more porous.

The following steps are almost completely routine: the smears are treated with a specific luminescent serum; the non-bound excess serum is decanted from the preparation, after which the latter is examined under a fluorescent microscope.

What then is unique about the immunofluorescence diagnosis of viral and rickettsial infections that distinguishes it from other immunological procedures used in diagnosis? Primarily, it is extraordinarily sensitive: for viruses, it measures up to or surpasses by an order of magnitude the

best of routine procedures, while for rickettsia, it surpasses the latter by 100-1000 times.

This remarkable sensitivity is demonstrated by the higher rate of positive results of immunofluorescence tests for viral and rickettsial antigens in patient secretions, as compared to any other technique. The diagnosis of poliomyelitis provides an example of this (Fig. 10).

Another illustrative example is the immunofluorescence diagnosis of influenza. During an influenza epidemics, the virus antigens were detectable in the samples of washings of the upper respiratory tract in 75 per cent of patients, and in periods between epidemics in 50 per cent of patients. These figures are unattainable with any of the other known tests.

The author has deliberately chosen, by way of example, the immunofluorescence diagnosis of influenza, because fluorescent antibody technique was widely applied in diagnosing acute viral respiratory diseases that resemble influenza. The detailed development of the immunofluorescence technique for the diagnosis of acute respiratory disease was carried out at the All-Union Research Institute of Influenza. The procedure need not be discussed in detail, but it is interesting to note that the most convenient preparations are nasal smears taken on cotton swabs after the nasal cav-

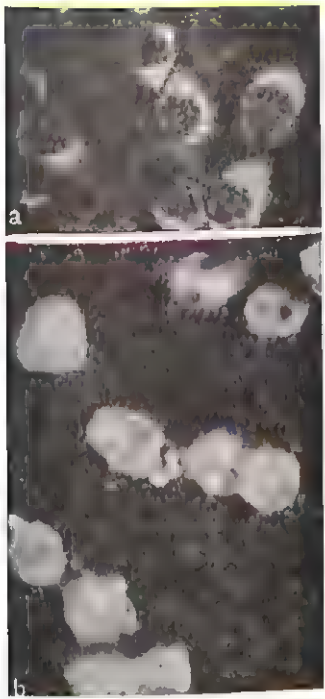


Fig. 10. The immunofluorescence picture of the poliomyelitis virus:
a—5 hours after contamination; b—24 hours after contamination

ity itself is first cleaned to remove all mucous secretions. The smears are taken within 24-28 hours from the onset of the disease. The swab is then soaked in buffered

physiologic saline solution, and after further concentration, a smear is prepared from the solution on a glass slide.

As early as 1956, the first reliable data obtained by immunofluorescence diagnostic investigation of influenza appeared. Since then, a considerable amount of new information has been accumulated, which provides further proof of the successful application of this method during epidemics. A Japanese study in 1965 demonstrated the presence of luminescent nasal epithelial cells in 80 per cent of patients by treating nasal smears with a luminescent serum. Still higher values (94%) were demonstrated in a study carried out in 1969 in Leningrad.

Diagnostic errors can occur because of the presence in the studied preparations of different types of luminescent epithelial cells that can provide evidence of the same disease with varying degrees of probability. However, the majority of investigators agree that the most valid evidence of acute viral respiratory disease is provided by columnar epithelial cells which can be further subdivided into ciliated, round, and ovoid cells (Fig. 11). Most often, the luminescent antigen accumulates only in the cytoplasm of these cells, although sometimes it can be seen in other cellular structures, and more rarely it is reduced to only the cell nucleus (Fig. 12).

Based on data from a study carried out on several thousand patients at the Insti-

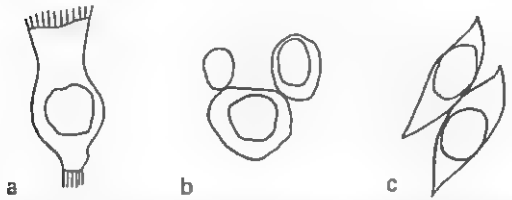


Fig. 11. Cells of the columnar epithelium extracted from the nasal secretions of patients suffering from influenza:
a—ciliated; *b*—round; *c*—ovoid

tute of Influenza, the correlation between the results of immunofluorescence analysis

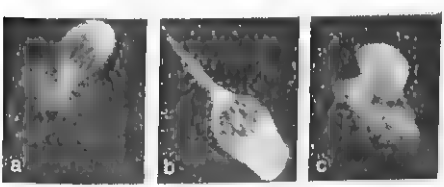


Fig. 12. Patterns of immunofluorescence staining of columnar epithelium in the nasal secretions of patients suffering from influenza:
a, *b*—ciliated cells with partial (*a*) and complete (*b*) pathogenic involvement of cytoplasm; *c*—contaminated (bright emission) and normal (weak emission) ovoid cells

and those obtained by other techniques reaches 90 per cent or more. This in itself provides a strong recommendation for the

application of the immunofluorescence technique to the diagnosis and prevention of epidemic diseases.

There is yet another possible application of immunofluorescence diagnosis: the detection of cutaneous viral and rickettsi-

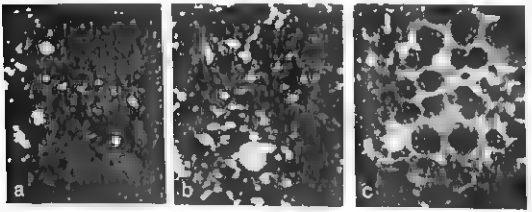


Fig. 13. Patterns of immunofluorescence staining of viruses (*a*) and Rickettsia (*b*, *c*):
a—extracellular pox viruses in vesicular fluid; *b*—Rickettsia in smears of yolk membranes; *c*—intracellular Rickettsia in the spleen of the mouse

al lesions. A high success rate was demonstrated in detecting pox virus in smear specimens taken from vesicular fluid or from drying skin crusts. The main advantage of this method is the rapidity of procedure: a definite answer can be ready within one or two hours. At the present time, a similar procedure for the detection of rickettsia is not so well developed. The positive results now available are mostly the result of experimental studies on infected animals such as mice (Fig. 13).

Finally, it must be emphasized that fluorescent antibody technique has almost limitless applications in medical and biological investigations. However, in the daily practice of general practitioner this simple and efficient method is still applied far too rarely.

Why is this so? The author agrees here with O. V. Baroyan, Member of the USSR Academy of Medical Sciences, who thinks that the reason is only a lack of knowledge about the possible applications of immunofluorescence in diagnosis of diseases.

Hopefully, this brief section will to a certain extent contribute towards making the fluorescent antibody technique more popular than it is now.

Investigations of Biological Membranes

The reader may recall that in biology a "membrane" is a thin layer of tissue forming the surface of cells and also the small tubes and vesicles of the endoplasmic reticulum of the cell cytoplasm. The main function of such membranes is to regulate the transport of ions, sugars, amino acids, and other metabolites.

Frequently, changes in biological membranes can lead to deranged functioning of the cell, which in turn leads to the develop-

ment of pathological conditions, which may be fatal to the organism. Cell membranes are so located that they are actually the first to encounter unfavourable conditions that may result from external factors or from intracellular processes. Cancer, mental disorders, cardiovascular diseases, neurological disorders, and hormonal imbalances are all diseases in which biological membranes are affected.

This is one aspect dealing with the function of membranes in pathological processes. The other aspect is the change in membrane permeability, as well as in other properties, which results from the ability of membranes to bind with most drugs, hormones, and toxins. In this way, the structure of the membrane is changed, as well as the activity of the whole system of membranes, which influences the cell on the whole.

Thus it is quite understandable, that one of the fundamental directions in modern biology is the study of the molecular structure and the function of biological membranes. Physical methods of investigation are responsible for most present knowledge about their structure and mechanism of action. Fluorescence analysis has played a prominent role in these studies.

Luminescent dyes were used for the first time in investigations on cell membrane in the late 1960s. These pioneering works were

undertaken by the Americans A. Azzi and B. Chance who, by observing the changes in fluorescent dyes (sometimes called fluorescent probes), studied the conformational restructuring of the membranes. In the early seventies such fluorescent dyes were first applied in this country in investigation that was begun at the Moscow Second Medical Institute. These dyes assisted in demonstrating the interactions between biologically active compounds and cellular and intracellular membranes, as well as the structural and functional membrane changes occurring in some pathological processes.

The unique advantages of fluorescent dyes, applied in a wide spectrum of biological studies, are described in a book written by Yu.A. Vladimirov and G.E. Dobretsov*. Among other topics, the following are discussed: functions of membranes in the transport of ions and the arising of surface potential; physical properties of lipid membrane components (phase state, viscosity changes, and the presence of polar molecules like H_2O); protein-lipid interactions stimulated by the membrane enzymatic systems.

Many of these problems can actually be dealt with by using other methods. The choice of a particular method is always motivated

* Vladimirov Yu. A., Dobretsov G. E. *Fluorescentnye zondy v issledovanii biologicheskikh membran* (Fluorescent Probes in the Study of Biological Membranes). Moscow, Nauka (1980).

by its availability. From this point of view, the method of fluorescent dyes has practically no equal. It is a generally available technique. In recent years, it has become easier to obtain a spectrofluorometer for a biochemical laboratory. Moreover, this fairly sophisticated device is frequently not required. A simple fluorometer and a set of light filters are usually sufficient.

The availability of the technique does not, however, guarantee easy interpretation of the results obtained by this technique. Before undertaking a series of experiments with fluorescent dyes, one must remember that the information thus obtained will be dependent, to a larger extent, on the chemical nature and properties of such dyes. According to their application, fluorescent dyes can be divided into three large groups.

1. Water-soluble (hydrophilic) compounds, like cyanine dyes and other compounds with ionic structures. Most characteristically, these dyes have a very high sensitivity to changes in the membrane potential and cell acidity. Water-soluble dyes are distributed mainly in the water medium surrounding the membrane, rather than in the membrane itself.

2. Hydrophobic dyes, such as condensed hydrocarbons, 3-methoxybenzanthrone and others. These are soluble mostly in the lipid components of the membrane, and

their fluorescence parameters (intensity, polarization, half-life) depend on the physical characteristics of the microenvironment of the probe in the membrane studied. Fluorescent dyes of this type are a very sensitive means for studying the physical properties of the inner content of the membrane.

3. Dyes that are distributed between water and membrane phases in a measurable ratio. A typical example of this type of luminescent dyes is anilinenaphthalenesulfo acid, which has already been mentioned in a previous chapter. The molecules of this compound become fluorescent only when bound to the membrane, thus being a sensitive indicator of transmembrane potential, ion transport across the membrane, membrane protein conformation, etc. The fluorescence of this type of dyes reflects almost every structural change taking place within the membrane which can result from various chemical or physical factors, including cooling or heating, as well as the actions of enzymes, drugs or hormones.

The classification outlined above is rather conventional, because some luminophore molecules are always present in the membrane, and some are always present in the surrounding water. The ratio between the two is the determining factor. Thus, the distribution coefficient between the membrane and water becomes the most important characteristic of the fluorescent dye.

Having acquainted the reader with the general principles of the applications of different dyes for the study of membranes, the applications of each type of dyes in medical practice will be discussed.

Let us begin with water-soluble dyes. Their application in cytodiagnosis has made it possible to assess the viability of mitochondria, based on the accumulation of the dye solution (4-(N-dimethylaminostyryl)-1-methylpyridine chloride) by the mitochondria. It was discovered that mitochondria treated by some poisons (for example, potassium cyanide) or large doses of certain drugs, such as oligomycin or dimedrol, lose most of their surface membrane potential and become unable to accumulate the molecules of the dye. Thus, in healthy white blood cells stained with a dye, fluorescence microscopy reveals mitochondria as bright spots, while in "poisoned" cells with dead mitochondria (which results in the respiratory failure of the organism) no such spots are visible.

In addition, water-soluble dyes are applicable in assessing membrane permeability, as is demonstrated by the presence of either the molecules of the dye itself, or the molecules that extinguish the fluorescence of the dye. The method is as follows: liposomes*

* Liposomes are models of biological membranes; they are spheres of lipid bilayers.

containing antigens on their surface, are filled with a concentrated aqueous luminophore solution in the presence of fluorescence extinguishers. Then antibodies and complement are added. The membrane structure then gradually starts to deteriorate and the dye emerges from liposomes, which increases the intensity of its fluorescence. The exit of the luminophore from the membrane is almost as rapid as that of glucose when the latter is used in studies on membrane permeability. The obvious advantage of the dye over glucose is the ease of recording its fluorescence.

Hydrophobic dyes probably have more prospects for future development in diagnostics than any of the other fluorescent dyes. Very interesting results were achieved when 3-methoxybenzanthrone was applied in identifying T cells and B cells. Approximately two decades ago the two main types of lymphoid cells were discovered in blood, namely thymus-dependent cells (T cells), and those produced by the bone marrow and responsible for antibody production (B cells).

In the healthy human organism the ratio of these two cell types is always the same: the T cell level in the blood varies between 50 and 70 per cent, and the B cell level, between 15 and 30 per cent.

Until recently, there were no simple techniques that could be used for the identification of T and B cells. Under an ordi-

nary light microscope they look almost identical; however, their luminescence is specific. The luminescence technique itself could not be simpler. A drop of a very dilute solution of 3-methoxybenzanthrone in an organic solvent—dimethylformamide is added to a drop of a suspension containing several million cells. The preparation is then placed under a fluorescent microscope, and the cells are examined for varying intensity of their green fluorescence. Numerous studies have indicated that in normal blood samples fluorescence intensity of T cells is 1.8-2 times lower than that of B cells. The assessment and identification of cells take no more than 15 min.

This difference in the intensity of fluorescence of the dye has two possible explanations: the different characteristics of T lymphocyte and B lymphocyte membranes, or the different amount of membranous material in these cells may be responsible.

Further and more detailed studies of T and B blood cells using luminescent stains, such as 3-methoxybenzanthrone, anilinenaphthalenesulfonic acid, or others, revealed that both of the factors mentioned above are responsible, although the second factor is most important. B cells were found to contain more membranous structures than T cells. It is supposed that in B cells, as compared to T cells, the area of intracellular and plasma membranes is greater.

The different binding of B cells and T cells to fluorescent dyes would have been of interest only to biologists, and not to physicians, if not for one phenomenon. In the last decade a series of studies revealed that the ratio of T lymphocytes to B lymphocytes is changad in quite a few pathologies, such as leukosis, various infections, autoimmune disorders like lupus erythematosus, and others. Any change in this ratio affects the general intensity of emission of 3-methoxybenzanthrone-stained lymphocytes. For example, the mean intensity of lymphocyte fluorescence in chronic leukosis is 2-2.5 times higher than normal, and in bronchial asthma it is 1.3-1.5 times lower than normal. This might be used as a reliable diagnostic sign.

However, hydrophobic dyes are not only used for the identification of B and T lymphocytes and for establishing a reliable diagnosis. Using 3-methoxybenzanthrone, the mechanism of action of nonachlazin, an antianginal drug developed in this country to control myocardial ischaemia, was investigated. Earlier physiological data indicated that nonachlazin plays a prominent role in catecholamine metabolism, in particular, it inhibits the feedback mechanism of noradrenalin transport across the membrane. The studies with 3-methoxybenzanthrone revealed a competition between nonachlazin and noradrenalin for

binding centres in the phospholipid membrane.

In addition, 3-methoxybenzathrone and other hydrophobic dyes can assess the affinity of many hormones, antibiotics and other biologically active compounds to membranes. Although this method of assessing the affinity to model membranes does not replace studies of true biological activity, it is still helpful in the preliminary selection of membranotropic drugs, i.e. those influencing membrane structure.

Finally, let us consider the third group of fluorescent dyes. Such stains have been found to be very sensitive to any changes in the cholesterol/phospholipid ratio. It is well known that a change in cholesterol level in cells and tissues occurs in various metabolic disorders, as well as in alimentary hypercholesterinemia, which is a high blood cholesterol level due to excessive fat intake. The role of cholesterol accumulation in the development of atherosclerosis has been discussed above. The reverse situation, that of a cholesterol deficiency, is generally associated with lymphoid cell malignancy. An increase in the cholesterol/phospholipid ratio leads to changes in membranes which actually cause a 2-fold increase in the fluorescence intensity of anilinenaphthalenesulfonic acid. Cholesterol accumulation also affects the fluorescence parameters of some hydrophobic probes, such as diphenylhexa-

triene and pyrene; however, the studies with anilinenaphthalenesulfonic acid gave the most impressive results.

The book by Yu.A. Vladimirov and G. E. Dobretsov discusses many other ways of applying fluorescent probes in diagnosis as well as in the assessment of disease severity and control of the course of the disease.

The previous chapters have given the reader a clear understanding of two main points, i.e. that fluorescence analysis has many unrivalled advantages, which provide many prospects for future applications of luminescence techniques in medical science and practice, and also that the practical realization of these advantages has made fluorescence analysis irreplaceable for such global problems as investigations on the physiological processes within the human organism, drug intake control, and medical diagnosis.

The discussion could end here, but for one consideration: one must remember that health care is based not only on a network of holiday homes and hotels, hospitals and in- and out-patient clinics, but also on specialized medical stations for sanitary and epidemiological control. The health of human beings also depends, to a large extent, on the "health" of the air, land and water, i.e. the "health" of the environment. Can fluorescence analysis also be useful in this area? It certainly can.

Luminescence and Food Quality Control

The importance of nutrition is beyond dispute. It has always been so, as the German proverb argues: "You are what you eat." Thus, one cannot be indifferent to the quality of what one eats. The quality assessment of food products by fluorescence analysis will be discussed below.

Foods of animal origin. What food products should we start with? The reader probably remembers the Latin expression *ab ovo*, which literally means "from the egg on", but is used figuratively, in the sense of "from the very beginning". This is just the way any literary, or, more precisely, any popular-science, work ought to be written. This recommendation is here taken literally and our discussion will begin with the luminescence quality control applied to chicken eggs.

Already in the thirties, an original method for assessing the egg quality without breaking the shell was developed. It was based on the fact that the luminescence of an egg's content is observable through the shell, and changes from red to light-blue, depending on the time and conditions of egg storage. Fluorescence analysis was found still more useful in identifying eggs infected with the luminescent bacteria *Pseudomonas fluorescens* (luminescent microorganisms will

be discussed in more detail later on). The pigment pyoverdin, which is produced by the multiplying bacteria, is so brightly luminescent that the egg shell presents no obstacle for observing the emission. Detailed investigation of these unusual bacteria revealed that at first, they form their colonies only on the inner surface of the shell, and after 1-8 days they penetrate the white of the egg, which then also becomes luminescent. When the egg white is totally luminescent, the amount of bacteria reaches the gigantic figure of 10^7-10^9 bacteria per 1g.

If the lunimescence quality control of food products were to be discussed chronologically, then one would have to begin not with eggs,. but rather with foods such as meat, fish, fats, and milk. The emission of these food products under UV light was observed in the pioneering investigations by the Soviet investigators—enthusiasts of luminescence techniques in food quality control—R.Ya. Gassul, I.M. Menshikov, and G.D. Leskov. They found that the colour and intensity of the natural luminescence of food changes after storage and deterioration in quality of the food products. Despite some variation in the results (the emission is strongly affected by any accidental admixtures and metabolic products of microorganisms), these first investigations indicated that this field has good prospects for development.

Today, fluorescence analysis is widely applied in public health not only for the quality control of food products, but also to detect traces of chemical preservatives, drugs, antioxidants, flavouring agents, pesticides, and food dyes.

The largest amount of literature on the luminescence quality control of foods is devoted in particular to fish and meat products. One of the first serious studies on the food quality of fish was done before World War II at the Institute of Nutrition of the USSR Academy of Medical Sciences. This study demonstrated that in some instances fluorescence analysis can detect fish spoilage in early stages when organoleptic methods are useless. The main conclusions drawn from this particular study were the following: (a) fresh fish is only very weakly luminescent; (b) early stages of fish spoilage are manifested by a light-blue emission on the surface and in the inner tissues of the fish; (c) an amber or red luminescence is evidence of a very advanced stage of spoilage; (d) cooking does not in any way affect the emission. What then is the reason for the luminescence of slightly spoiled fish? Most investigators believe that it is probably due to luminescent microorganisms that invade the decaying tissues.

At this point, a short digression is necessary to explain that biological luminescence is a phenomenon which is widespread in

organic world. The reader probably remembers wondering as a child about luminous fire-flies, or pieces of rotten wood. Although biological luminescence was first described in the literature in 1742, some luminous animals had already been mentioned long before that in the writings of Aristotle (384-322 BC) and Pliny (23-79 BC). Biological luminescence has been observed not only in bacteria (*Pseudomonas*, *Bact. Phosphoreus*), but also in fungi (*Armillaria mellea*, *Pleurotus olearius*, etc.), in some higher plants during the period of growth, and in many animals (crustaceans, insects, etc.). According to its origin, biological luminescence is a typical example of chemiluminescence where the energy is supplied from the oxidation reaction with participation of oxygen.

Let us take a closer look at how the phenomenon of natural luminosity was investigated by different scientists. At the end of the last century, Raphael Dubois, a French physiologist from Lyons, studied the mechanisms of light emission by click beetles. He put the luminescing organs of these beetles in a mortar, added a small amount of cold water, and ground them; the resulting mixture luminesced for several minutes. The emission ceased much sooner if the water had been deoxidised and the organs were ground in the absence of air. When hot water was taken for the experiment, no

emission was observable. But, when the warm mixture was added to a cold one that had ceased to emit light, the resulting mixture produced luminescence once again.

Thus, Dubois came to the conclusion that, in addition to water and oxygen of the air, there were two other compounds that participated in the luminescence reaction. These compounds found in the organs of beetles had a different reaction to hot water, and Dubois invented special names for these substances. The one that was stable to heat (the oxidized one) was called luciferin; the unstable one (the oxidizing enzyme) was called luciferase.

More than half a century passed before the mechanisms of bioluminosity became the object of interest for a group from the John Hopkins University. Their investigations demonstrated that the conditions for luminescence described by Dubois were indeed necessary, but not at all sufficient. Emission also requires a large supply of energy in the form of adenosine triphosphoric acid (ATP). This discovery was made exactly at the time when the prominent role of ATP in many biological processes was becoming more and more evident. It was found to be essential for protein metabolism, for carbohydrate metabolism in cerebral tissues, and most importantly for the muscular activity of the body. ATP molecules can also interact with molecules of myosin,

the contractile protein in muscle, resulting in the production of energy. It is this chemical energy that is responsible for the strength and activity of the muscular system.

Before returning to the topic discussed earlier, there are a few more things to say concerning one very interesting experiment. Several years ago it was suggested that a special device that could detect the existence of life on other planets be constructed. The reader may ask what relationship bioluminescence has to the experiment. The answer is simple: the head part of this device was to include a mixture of luciferin and luciferase and to be equipped with a light-recording mechanism. If this device, upon encountering a celestial body, should detect and record an emission, then one could logically conclude that ATP is present and, consequently, that life similar to that on earth is present. What is left is only to wait for the results of such an experiment.

Now, let us return to the fluorescence analysis of food quality. The luminescence control of meat products has the same limitations as those of fish, namely, the variation in emission. Usually, muscle tissues of fresh meat are not luminescent at all, while connective tissues emit a light-blue glow. In storage, after the meat has begun to spoil, the formerly dark areas

start emitting as a result of bacterial activity and enzymatic-oxidation reactions. An absolute indicator that meat has become inedible is the appearance of a red glow, which is characteristic of porphyrins which are the decomposition products of hemoglobin and other analogous compounds. The appearance of differently coloured luminescent spots on the surface of stored meat indicates the presence of microorganisms, moulds, and fungi.

Here is one example of such a study on the luminescent properties of beef and pork, fresh, as well as infected by the tapeworms *Taenia solium* and *Taeniarhynchus saginatus*. It was demonstrated that the infected meat exhibits bright red emission. This emission, as close examination revealed, was caused by the tapeworm larvae, the so-called cysticerci (*Cysticercus cellulosae* and *Cysticercus bovis*). Interestingly, the luminescence was not emitted by the larvae themselves, but by the fluid in a bladder-like cyst of parasites. Such differences in the luminescence of fresh and infected meat can be used to determine whether or not the meat is fit for use.

It was known before this study was undertaken that cysticerci can be destroyed by freezing, pickling, or by heating at 60 °C for a long period. Unfortunately, the investigators did not succeed in determining a difference between the luminescence of

active and dead parasites. This would have provided the basis for the development of an easy and quick technique for the assessment of cysticerci viability, and, thus, of the degree of meat decontamination.

Research on this problem has not been limited only to studies of meat luminescence. Attention has also been focused on the juice of meat and cooked meat dishes. The luminescence spectra of the extracts of fresh and spoiled meat were found to be markedly different: the latter showed a displacement of the absorption maxima towards the long-wave region. The longer the meat had been stored, the more intense was its luminescence; its colour also changed from greenish to milky-blue.

Fluorescence analysis is often used for checking cow milk. It was noticed long ago that the luminescence of fresh milk excited by UV light is greenish or amber-brown. This is due to the presence of riboflavin (vitamin B_2), a biologically active polycyclic compound.

If the public health worker responsible for the quality control of food notices any unusual changes in the colour of the milk luminescence, he or she must immediately perform a more detailed bacteriological and microscopical investigation of the milk. What can cause such changes? Among the possible reasons is the souring of the milk. In an acidic medium (more precisely,

at pH < 4), riboflavin ceases to emit light. In addition, when milk has been stored for a period of time, some of its components, such as amino acids and sugars, unavoidably start to interact. A change in the colour and intensity of the emission allows one to discover such processes long before the milk itself displays any visible changes, such as turning brown in colour. If milk has been illuminated or simply kept in a well-lighted room for a long period of time, its emission acquires a bluish tint. And, if the emission is pale yellow in colour, one can suspect an udder disease in the cows the milk was taken from.

In recent years, veterinarians have widely used a variety of antibiotics for curing live stock, especially for treating mastitis in cows. On one hand, this is beneficial because there are really no any other agents as effective as antibiotics; but there is also another side to the story. The problem is that antibiotics can get into milk. Why is this harmful? First of all, it has been shown statistically that the intake of drugs among the population is high as it is, and, thus, any additional doses are quite unnecessary. Second, traces of antibiotics can inhibit the growth of curdling bacteria in milk, thereby interfering with the technological processes of cheese-making.

Until quite recently, antibiotics in milk were detected by routine techniques, which

also included fluorescence analysis. The latter, as was already mentioned, was based on the reaction with luminescent aliphatic amines. However, not long ago a new method was developed, which is more sensitive, rapid, and original. A mixture of luminophore substances, consisting of fluorescein and its sodium salt, uranine, is added to the antibiotic, which is then administered to the ill cows.

If the milk of the cow that had received such an agent displays a positive reaction to antibiotics, then it is certain that it contains fluorescein, which will impact characteristic emission to the milk. The higher the concentration of the drug, the greater is also the intensity of luminescence.

The luminescence of butter, oil and fat, in contrast to that of cow's milk, does not reveal anything about the quality and freshness of these foods. However, despite this drawback, fluorescence analysis has proved to be an efficient means of detecting any admixtures or contaminants in the food. In 1928, using fluorescence analysis, it was already possible to detect the difference between pure butter and butter containing 8 per cent of margarine. Foreign traders probably did not know about this: some of them still now attempt to profit by selling butter with margarine at the price of the pure product.

Most often the quality of butter and vege-

table oils is spoiled by contamination with mineral oils. Although they may be present in very small amounts, sometimes even less than 1-2%, this is exactly the case when the rotten apple spoils the bunch. Fortunately, mineral oils can be easily detected, even in such low concentrations, because of their characteristic emission.

More than three decades ago, the German investigator F. Schenberg developed an interesting and promising method for evaluating the freshness of fat. The procedure is amazingly simple: fat samples are treated with a very dilute solution (a 0.1% solution) of luminescent dye Congo red. The resulting luminescence varies depending on the freshness of the original material. Unfortunately, this technique has not yet become popular in industry.

Vegetable products. Luminescence technique for the quality control of food products have been most effectively applied to vegetables, fruits, vegetable oils, etc. The applicability of luminescence technique will here be demonstrated using the control of the quality of potato.

In the early fifties, V.N. Gyrenko and M.I. Golland from Leningrad suggested an original method for determining whether or not potatoes are diseased. The need to develop such a new and sensitive technique was most urgent. The traditional method consists of simple visual examination and,

thus, depends on the personal approach of the agricultural worker or dealer, leading to biased results. In addition, even the most careful examination cannot reveal that the product is infected during earliest stages of disease because the signs appearing on the surface of potatoes are invisible in daylight.

The new method that was developed in Leningrad, is based on the examination of the potato under UV light. This method has none of these shortcomings. The researchers began by observing the emission of healthy potatoes. It turned out that the colour varied for different varieties of potato. Thus, sections of the "Kameraz" potato emitted a bright amber colour; those of "Kalev", grey-amber; and those of "Berlichengen", greyish-brownish. A total of 70 varieties of potato were examined in this manner. The pattern of luminescence drastically changed if potatoes were frostbitten, or affected by phytophthora. The frostbitten potato revealed a light-blue luminescence, the affected potato, bright blue.

In this way luminescence method allows for the rapid and timely evaluation of potatoes, which can then provide the basis for deciding whether the product is of high quality and fit for prolonged storage.

Thus, the method is simple and convenient and, it follows, should be applied as a standard routine procedure at places where vegetables are stored. This was realized at

once in Leningrad, and a number of fruit and vegetable processing plants quickly applied luminescence to the quality control of potatoes. The economic effect was immediately evident: there was a 1.5-3-fold increase in the percentage of bad potatoes.

However, the wide application of this new method required more information on the luminescent properties of potatoes beyond that concerning the frostbitten tubers or those infected with phytophthora. A new extensive study was therefore undertaken, to investigate other diseases that affect potatoes.

It was found, for example, that only the affected areas of tubers infected by *Phizoctonia* fungi luminesce. Potatoes from "Berlichengen" plant that is affected by the wrinkle mosaic virus emit a whitish-blue emission that extends from peripheral regions of section to the centre. Irrespective of their variety, potato tubers that have sprouted in the dark luminesce in the same way as tubers that have not sprouted. And if the sprouting occurs in the light, thin layer can be noticed under the peel that gives orange luminescence.

But why have only potatoes been discussed? Is it possible to evaluate the quality of other vegetables using luminescence techniques? It turns out that it is.

One of the earliest observations by researchers demonstrated that changes in the

luminescence of cucumbers, beans, and green and red cabbage reveal the beginning of spoilage at stages still unobservable by other methods. This type of examination was also done during canning with a noticeable effect: defects in canned products were reduced by more than half.

However, infection with the mosaic virus in cucumbers, onions, and other such vegetables, proved to be more difficult to detect, because the natural emission of their cut sections was too weak for adequate differentiation. Treatment with a luminophore was necessary, with subsequent examination of the stained specimens under a fluorescent microscope. Four types of luminescent dyes were tried, and a 0.1% aqueous solution of acridine orange provided the best results. After such treatment of the vegetable sections, the viral inclusions emitted a bright green colour, while the cell nuclei were coloured greenish-blue.

Of course, such techniques are too complicated to be applied at agricultural storage stations. However, luminescence techniques for the identification of viral diseases are of great interest in the agricultural sciences.

Although fluorescence analysis is efficient in the quality control of vegetables, it has been more widely applied in the quality control of fruit, especially of citric fruits such as tangerines, oranges, and lemons. This is easy because these fruits ripen under

the hot southern sun. What, however, is the connection with luminescence analysis? The reader may remember that in order to excite luminescence a UV source is necessary. Usually, this source is a mercury vapour lamp fitted with light filter that separates the visible part of the light spectrum. Sunlight includes, along with the visible part, IR and UV regions. Geographically, the lower the latitude, the more UV light there is that reaches the earth's surface. This is, by the way, the reason for the so highly praised "southern" tan.

Thus, if the southern sunlight is transmitted through the filters, the isolated UV radiation is quite sufficient for excitation of luminescence. According to this principle, the first specialized laboratory for the fluorescence analysis of fruit was constructed in Yalta (the Crimea) more than 30 years ago. The windows in the laboratory were not fitted with ordinary glass; instead light filters were installed.

The studies undertaken in this laboratory were not, however, the first ones aimed at this specific problem. A luminescence technique for evaluating the quality of oranges and tangerines had been developed earlier in the northern city of Leningrad. V. N. Gyrenko and M.I. Golland, who were already mentioned in the discussion on the fluorescence analysis of potato, conducted this research. They designed a special apparatus

that was installed at a Leningrad fruit canning factory. It consisted of a wooden cabinet painted black on the inside and a UV source (quartz-mercury lamp) fixed at the top, which illuminated a moving cart loaded with fruit. This simple apparatus provided an easy and rapid procedure for sorting fruit according to quality, and selecting unspoiled fruit for longer storage. Most important again, was that fluorescence analysis allowed for the early identification of fruit that was diseased but still looked perfectly normal in daylight.

Is there any difference between the properties of healthy and diseased fruit under UV light? There is, and the difference is manifested in the following way. Normally, tangerines emit a dark orange luminescence, however, should the tangerine be infected with *Penicillium italicum*, a blue mould, the centre of the affected area begins to emit a dark blue glow with a distinct light blue border, surrounded by a wide yellow ring. Frostbitten tangerines are recognized by the appearance of light blue spots against a dark orange background.

Oranges behave similarly: rotten areas are marked by the presence of light luminous spots against a dark background. Areas with brown patch have bright violet-blue glow; areas infected with black mould *Alternaria citri* emit dark olive luminescence. Very similar results concerning lumi-

nescent properties were obtained with lemons.

The simplicity and availability of the new method of sorting fruit has made it quite popular not only at warehouses, but also at canning factories during the primary processing of fruit.

As far as citric fruits are concerned, another unique application of fluorescence analysis must be mentioned, namely, the identification and quantitative assessment of admixtures in lemon oil. Since 1958 fluorescence analysis has been used abroad to detect whether or not cheaper admixtures have been added to this expensive oil.

As it turned out, once a large consignment of lemon oil that was ready for sale was analysed, and it emitted a glow that was markedly brighter than that of the original pure product. Chemical analysis revealed the presence of various coumarin derivatives. This particular oil turned out to be a mixture of lemon oil and the cheaper grapefruit oil. The latter contains the brightly luminescent 7-hydroxycoumarin (umbelliferone), which was mentioned previously. Pure lemon oil, on the contrary, contains only non-luminescent hydroxycoumarines. The presence of grapefruit umbelliferone can be detected in lemon oil at concentrations even less than 0.5 per cent.

This type of analysis has been called "sorting", by Academician N. I. Vavilov.

It can be applied to the detection of the purity of a large number of different food products, and is by no means limited only to the example described above. The detection of margarine admixture in butter, which was already mentioned, is another example, and the list can be added to.

The quality of flour and grain can also be checked by this method. Different varieties can be recognized by the different colour of their emission. The presence of any foreign material can also be detected. Flour usually emits a blue glow of various shades. The presence of fungal or bacterial colonies can change the colour of luminescence. *Bacillus sporogenes* emit a red glow; *Proteus* gives light blue glow; and *E. coli*, a green glow. Fluorescence analysis can also reveal infestation by field pests or moulds, as well as the presence of any traces of insectofungicides. Thus, if the grain is ripe, normal, and has no defects, the colour of its luminescence is blue. Any traces of moulds, or pests change the colour and increase the intensity of luminescence. Damp grain luminesces with an amber colour.

There is still more to say about the applications of fluorescence analysis for identifying flour or seed grains, evaluating their viability, and other purposes. However, these important and interesting problems belong to the domain of agricultural science, rather than to the field of public health.

Finally, it is time to discuss the most up-to-date method for the quality control of food products, which is based on the immunofluorescence phenomenon. In the early sixties, three general trends developed in the practical application of the fluorescent antibody technique. The first was the detection of pathogenic organisms in food products, for example, Salmonella*, which is present in raw meat and poultry, as well as in cooked products. This method is not more sensitive than standard bacteriological technique. Its advantage lies in the small amount of the time required to perform the technique.

Since 1972 the immunofluorescence detection of Salmonella in food products has been widely applied abroad, in particular in the USA, when the procedure was greatly simplified by the development of the "Fluoro-Kit", a special laboratory kit designed for this analysis. The kit contains all the equipment and reagents needed to perform 100 tests on food products, namely, glass slides, fixatives, washing fluids, a pipette syringe for injecting the luminescent serum, and so on. Along with the rapidity of the procedure (results are obtainable within 18 hours, after the specimens have been cultured in a special broth, by observing lu-

* Salmonella are non-sporogenous bacteria including more than 20 different types, among which there are typhoid and paratyphoid bacteria.

minescent Salmonella cells through a fluorescent microscope, whereas standard techniques require 5-7 days), there are other advantages. The technique is economical (only 1 ml of the luminescent serum is required to perform 100 tests), and also the kit itself is portable and quite handy. Thus, the microbiological technique can be performed anywhere without any special laboratory conditions, and this makes the overall testing of large consignments of food products convenient.

This first trend in the application of immunofluorescence technique is not limited only to the detection of Salmonella. This technique has also been shown to be reliable for the identification of dysentery bacteria in milk and water, and also of *Staphylococcus aureus* in dry defatted milk.

The second trend in the application of this method was directed specifically at the detection of microbial contamination in the food industry. Fundamental contributions to this field were made by the Austrian scientist Klauschofer who in 1966 conducted investigations on the detection of alien microflora in a number of food processing plants, especially sugar factories. A highly effective method for the detection of the thermophilic bacteria *Bac. stearothermophilus* in sugar syrup was developed. This bacteria can result in a considerable loss in the sugar product. Somewhat later, another group of

investigators suggested immunofluorescence method for the control of yeast microflora; this method is indispensable in detection of contamination of beer and alcoholic products with infectious organisms.

And, finally, the third trend was the luminescent control of the propagation of beneficial microflora employed in some food industries. A series of microbiological studies on fermentation, the most important stage in bread-making, serve as a good illustration of the possible applications of fluorescence analysis in this direction. The immunofluorescence technique made it possible to reveal the origin of yeast microflora in rye dough fermentation, an otherwise complex problem. Thus, it was established which species of lactic acid bacteria take the most active part in this kind of fermentation, and their development was followed throughout all the stages of this long process.

Many other methods that rely on luminescent antibodies and that are applied in the quality control of food products could be mentioned. However, the author now wishes to fulfill a promise to discuss the role of luminescence in yet another important field, the control of air and water pollution.

Pollution Control

The first attempts to find any correlation between the luminescence of water and the degree of its purity were made in early 1930s. Already at that time the problem of water pollution was becoming rather disturbing to scientists, although nobody as yet was bold enough to openly call the rivers, seas and oceans "the garbage heaps of human civilization". The very first observations revealed the existence of a direct correlation between the purity of water and the intensity of its luminescence. Actually, almost no emission was observed under UV light in water that had been carefully distilled or taken from deep underground wells. But regular water (taken from a water tap, or from a lake or a river) had a more or less intense emission.

What might be the cause of these differences? Obviously, natural non-distilled water contains certain luminescent admixtures that are either dissolved or are in suspension. Among inorganic compounds, only salts of uranyl UO_2^{2+}, platinocyanide salts of the type $K_2Pt(CN)_4$, and some compounds of rare-earth elements have been shown to display a relatively noticeable emission. Natural water contains either negligibly small amounts of these compounds, or none at all. Thus, the lumi-

nescence of any non-purified water can be caused only by organic compounds.

Proof of this is the proportionality between the emission intensity and permanganate oxidizability of water. Permanganate oxidizability is a very popular sanitation method for evaluating the concentration of organic compounds in water, based on their oxidation by potassium permanganate.

Fluorescence technique thus can be regarded as a reliable method of identifying organic compounds in water. However, its advantages are not limited only to this particular application. Close examination and careful assessment of the colour of the emission of the water and then, that of ether and chloroform water extracts, makes it possible to identify the origin of the organic compounds contaminating the water sources, as well as the degree of contamination.

Most often, water is contaminated by admixed humic compounds. These compounds are the main component of humus; in fact, *humus* in Latin means soil. Humic compounds are actually responsible for the characteristically bright luminescence of water in woodland and marsh areas. These compounds are luminescent because of their chemical structure: they are derivatives of phenolcarbonic acids and are formed by the aerobic decomposition of plant residue.

Other typical contaminating admixtures are organic compounds of animal origin,

domestic and industrial liquid waste, and fuel oil products.

The possible role of the so-called luminescence extinction phenomenon must not be overlooked in any quantitative assessment of water contamination. This complicated phenomenon may be caused by the presence of salts of iron, cobalt, nickel, halogens, mineral acids, phenols, aniline dyes, and other such compounds in concentrations greater than 150 mg/l. An excess concentration of organic compounds in the water may also contribute to extinction (concentrational extinction).

Specialists in the luminescence quality control of drinking water have not limited themselves to general theoretical recommendations, but have suggested a specific and easily available methodology for analysing water. The procedure itself is quite uncomplicated. A JI-80 apparatus, manufactured in the Soviet Union, a set of glass or quartz test tubes, and an attentive and intelligent experimenter are all that is required.

The UV source in the JI-80 apparatus is a ПРК-4 quartz-mercury lamp, with a light filter transmitting a light beam between 320 and 400 nm (the "nearest" UV region). The luminescence excited by this wavelength region is mostly due to organic compounds. The choice of this UV region as a source of excitation is not accidental. First of all, UV light with the wavelength

exceeding 320 nm does not induce any noticeable decomposition of organic compounds. (As the reader may remember, the shorter the wavelength, the higher is the light energy, as well as the possibility that complex molecules will be decomposed.) The second consideration is economical: the water luminescence excited by a source with this limited spectral region, can be observed in ordinary glass test tubes; it does not require the use of more expensive quartz tubes. To perform the analysis, water samples are poured out into test tubes, and the emission is observed throughout the whole water column, as well as in the meniscus. The meniscus often emits even when the column itself looks dark.

When is the application of the fluorescent analysis to the evaluation of water potability most reasonable? It is most useful when, for example, one must quickly choose from a number of water sources the one that is least contaminated, or for the control of water purification systems and identification of any connections between the water system and the suspected source of contamination.

The latter problem is approached in the following manner: a 2% fluorescein solution is injected into the source of contamination. If there is really a connection to the main water reservoir, its water will acquire a green luminescence that will be visible even if

the fluorescein concentration is extremely small.

A novelty in the practice of sanitary bacteriology is the use of luminescent antibody technique. Several years ago the use of nonluminescent membranous filters was introduced abroad for the identification of *Brucella* and other pathogenic organisms present in water. The advantages of this immunofluorescence technique over traditional, bacteriological techniques applied in sanitary control of water were evident when these filters were cultured in lactose broth during 2-3 hours.

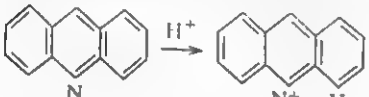
In recent years, specialists in the field of environmental control have been focusing more attention on the problem of the acidity of natural water sources. This problem is closely associated with another one, that of the so-called acid rain. Before discussing the problem, the physical and chemical methods of assessing the degree of acidity will be touched upon.

Acid-base indicators are used for the crude evaluation of acidity of liquid media. These indicators are usually phenolphthalein and litmus. The acidity control procedure is based on the dependence of the indicator's colour on the pH of the medium. For example, phenolphthalein, which is colourless in neutral or acidic media, turns crimson in alkaline solutions; while litmus displays a gradation from red to blue as the pH increases

from 1 (acidic medium) to 13 or 14 (strong alkaline medium).

More problematic are cases when it is necessary to measure the acidity of coloured liquids, such as wine. In this case, luminescent pH-indicators are required, which reflect any change in the acidity by a change in either the colour, or the intensity of their emission. The former are indicators such as acridine; the latter, fluorescein.

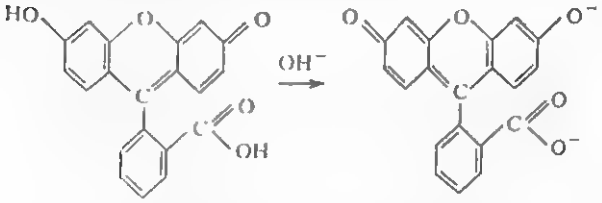
As the acidity of an acridine solution is changed from 6.6 to 4.8, the colour of its fluorescence changes from violet to bluish-green, which reflects the displacement of the fluorescence maximum from 440 to 490 nm. This change in the colour is caused by the change in the acridine molecule. In alkaline, neutral or weakly acidic solutions, the molecule is present in form I; in strong acidic medium, in form II:



I II

The picture is different when fluorescein is applied as an indicator. The emission of fluorescein is always green, with a maximum at 525 nm, irrespective of whether or not the medium is acidic, neutral or alkaline. However, when the medium becomes more alkaline, i.e. if the pH is increased, the inten-

sity of the emission also increases. This is associated with the transformation of the fluorescein from molecular form into an anion one; the latter form being the principal source of the emission:



The method based on measuring the pH of coloured solutions is applicable not only in the analysis of wine; sometimes, natural water may be so "thick" with various admixed components, that traditional acid-base indicators do not work in such a medium.

Now, let us return to the discussion about acid rain. This problem was first encountered in Norway in the mid 1950s. At that time it was noticed that the number of fish in their lakes was constantly decreasing. The reason for this alarming phenomenon was found to be acids contained in rainwater. This had a fatal effect upon the viability of the fauna that normally existed in fresh water reservoirs. The source of this high degree of acidity was found to be gaseous components of the atmosphere containing sulphur and nitrogen oxides, which

were released by heat and electric power plants and industrial plants.

To what extent does the acidity of contaminated rainwater deviate from normal? The answer to this question requires, first of all, a precise definition of what can be regarded as the normal value. It was found that even in the cleanest air found in the atmosphere, rainwater is characterized by a pH somewhat less than 7. The reason for this is that atmospheric carbon dioxide dissolves in water and acidifies the rainwater (the pH becomes 5.6-5.7). When sulphur and nitrogen oxides are dissolved in rainwater, a further drastic increase in the acidity occurs, with the pH decreasing to approximately 3-4. Acid rain can often be observed in the northeastern regions of the USA, and also in the industrial regions of Great Britain. When comparing water with pH of 5.6 to that with pH of 3.6, one should not forget that the acidity of the latter is 100 times greater than that of the former, instead of only 1.5 times, as it might appear to the layman (the pH scale is logarithmic!).

What happens when acidic rainwater gets into a freshwater reservoir? Normally, the water in ponds or lakes is weakly alkaline with pH up to 8, because of the presence of cations such as NH_4^+ or Ca^{2+}, which are either the products of organic decomposition, or are washed out of the soil.

At first contact with natural water, acid rain is completely neutralized. The duration of this process depends on the local soil components and their properties, but obviously, the neutralization cannot continue infinitely. Sooner or later natural water becomes acidified, thus threatening the survival of the local flora and fauna.

It is enough to explain that at pH of 6.6 snails begin to perish; at pH of 6.0, shrimps disappear completely and fish stop spawning. At pH lower than 5.5 many species of fish begin to die off, including pikes and perches. After a further decrease in the pH, sphagnum, a land moss, appears. It is inedible for the fauna, and has the ability to extract calcium from the water. At pH of 4.5, the chemical composition of the water becomes such that fish or frogs can no longer survive.

Returning to the Norwegian reservoirs, let us try to understand why Scandinavia first became subject to acid rain. The fact is that the rain in Scandinavian countries originates far beyond the peninsula, in the cyclones forming above the Atlantic Ocean. The cyclones then pass over the industrial basins in Britain and North Europe, where they absorb many tons of oxides. In the mountains of Scandinavia these humid air masses fall as acid rain. Almost 60 thousand tons of sulphur fall on Norway every year; 75 per cent of this is of foreign origin.

This unfortunate situation can be also seen in America, in areas downwind of large industrial centres. Acid rain is a regular phenomenon in the states east of the Mississippi.

How can this problem be dealt with? Obviously, the release of sulphur and nitrogen oxides into the atmosphere must be reduced. This is the most radical solution to the problem, and is far from easy. Waste purification facilities are very expensive. In the USA, only ten per cent of all heat and electric power plants consuming coal have such attachments. Draft bills calling for reductions in the discharge of sulphur dioxide into the atmosphere have been on the Senate shelf for years, and are still unlikely to be endorsed.

In this respect, the situation in Europe is somewhat more optimistic. Not long ago, in November 1979, the European conference on cooperation in environmental control was held. The resulting document was a convention on transborder long-distance atmospheric pollution, signed by the representatives of 34 European countries. The convention proposed a number of practical measures to detect the areas affected by acid rain, and to identify and remove their sources.

Returning to the luminescence control of environment, fluorescence analysis has been effectively applied for many years to assess

the degree of contamination of natural water by fuel oil and petroleum products. The necessity for this type of control has become more and more evident. Oil leaks in offshore drilling alone result in a waste output of about 30 million tons of oil every year.

The quantitative analysis of petroleum product in water is normally done by comparing the luminescence intensity of chloroform water extracts with that of standards containing known concentrations of oil. The colour and intensity of emission of petroleum products allow one to determine their origin, composition and purity. This method is very convenient for the identification of diesel and lubricating oils that have been illegally discharged into sea harbours by ships and boats. The importance of such a procedure becomes obvious from the fact that every year more than 12 million tons of oil, resulting from cleaning of the tanks of oil-carriers, and also from other causes of oil loss, are dumped into the seas and oceans.

People have become used to the fact that the water obtained from water taps must have been purified somewhere. Despite the fact that water processing cycles have been introduced almost everywhere in industry, water purification systems have been, are, and will be efficiently working to produce drinking water. The purification of atmospheric air is quite a different problem. Who

will be responsible for the purification of this most essential element? Civilization must learn to keep the atmosphere free from pollution. Today, policemen positioned on the main traffic arteries in New York have to wear gas masks; will this soon become a requirement for the militia in Moscow and Leningrad?

We can only hope that this will not happen and there are some grounds for hope. In the USSR, in spite of the rapid increase in traffic and industrial development, air pollution, on the whole, has been kept under control. The general objective today is to reverse the situation, i.e. to reduce the level of air pollution. To achieve this, it is absolutely essential to decrease the industrial release of chemicals to levels below a reasonable established limit.

Obviously, very sensitive techniques are needed to check the concentration of chemicals in air and since fluorescence analysis is outstanding for its sensitivity, it cannot be done without. The application of the fluorescence analysis in this field has indeed been quite effective.

The majority of methods for controlling air pollution have been developed specifically for control in industrial buildings. This is understandable, because one of the most important tasks is the safety of people employed in hazardous industries. Fluorescence analysis has already been applied

for more than 20 years to identify oil fogs (by the emission of mineral oil in dichloroethane), products of fuel oil pyrolysis, benzanthrone, acetylene, naphthylamines, boric acid, aluminium, and phthalic acid derivatives.

The identification of the last of these compounds should be discussed in particular, because it provides a very illustrative example of how tricky and complicated the actual performance of fluorescence analysis can be. The quantitative determination of phthalic acid derivatives requires at least three preliminary stages of chemical transformations. The first stage consists in transforming these derivatives into free phthalic acid; the second stage is the transformation of the acid into phthalic anhydride; and at the third stage this final compound is fused with resorcinol to produce fluorescein. The phthalate concentration in air is assessed by the intensity of fluorescein emission.

The compounds listed above can be quantitatively assessed in the air of industrial buildings. The list of the compounds that can be identified only qualitatively is much longer. These are the following: non-metals and their derivatives, namely fluorine, bromine, ozone, boron, arsenic trioxide, sulphur dioxide; many organic compounds, such as acrolein, aliphatic amines, nitrobenzene, hexachloran, formaldehyde; and

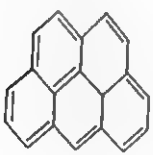
some metals, such as beryllium, tin, lead, zinc.

The fluorescence analysis of carcinogenic compounds is important enough to be discussed separately and at length. These substances (whose name is derived from the Greek *karkinos* meaning cancer or crab, and *genes* meaning bearing) have interested physicians for a long time, and the reason for this interest was totally unfortunate circumstances.

In 1775, Percival Pott, an English physician, described the cancer of chimney-sweeps. This type of malignancy affected the skin of the scrotum and was seen in boys who had been employed to clean old narrow flues. Cancer usually developed 10-20 years or more after the first contact of the patient's skin with soot, which contained coal tar. Occupational cancers were later discovered to result not only from contact with coal products, but also with goudron, the heaviest fraction of oil distillation. This explains the development of chronic and cutaneous lesions of the hands, the arms and the scrotum of workers occupied in oil processing industries and in asphalt road works.

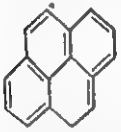
The incidence of occupational cancers caused by the direct contact of skin with goudron or heavy lubricating oils has been reduced almost to zero by the introduction of compulsory hygienic measures.

At present, the products of coal processing have been most studied among all the known carcinogenic compounds. The earliest evidence documenting the highly carcinogenic properties of coal tar was obtained by Yamagiwa and Ishikawa in 1917 in Japan. By applying this tar to the ears of rabbits, they stimulated the development of malignancies. Later it was shown that only some of the coal tar components are carcinogenic. The latter data were published in 1933 in Germany by Higer. The amount of work done in the course of his investigations might be equal only to the extraction of radium from uranium ore by Marie and Pierre Curie. Higer systematically fractionated 12 tons of coal tar and extracted a small amount of pure polycyclic hydrocarbons, some of which were highly carcinogenic agents. All of these substances were aromatic compounds, differing structurally in the number and sequence of benzene rings. Hydrocarbons with five-membered rings had the highest carcinogenic activity, the champion among them being the so-called 3,4-benzpyrene:



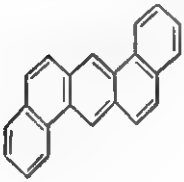
It must be stressed once again, that carcinogenic activity is to a great extent depen-

dent upon the bonding sequence of the benzene rings. Benzpyrenes generally consist of five rings, and thus, they can be regarded as derivatives of pyrene



a non-carcinogenic hydrocarbon, formed by four rings. Whether a five-ring compound will be carcinogenic depends upon the way the fifth ring is bound. If, for example, the five benzene rings are bound into the following structure

the result is the mildly carcinogenic 1,2-benzpyrene. But if, in addition to binding this fifth ring, the whole structure of the pyrene molecule is transformed, the result is the formation of 1,2,5,6-dibenzanthracene



which is only slightly less carcinogenic than 3,4-benzpyrene.

The first investigators who studied the effect of carcinogenic agents on living organisms encountered serious difficulties. The main problem then was the lack of a simple, sensitive and specific method for identifying and quantitatively assessing polycyclic hydrocarbons in tissues as well as in the environment. Naturally, scientists tried fluorescence analysis to overcome some of the difficulties. Already in 1927 the luminescence of coal tars was investigated in detail and found to be very intense. Carcinogenic hydrocarbons that were extracted in the pure form also gave intense luminescence.

It seemed that they could be easily identified by their specific emission. However, experimental studies with animals revealed that only a few of the large number of brightly luminescent analogous hydrocarbons were carcinogenic. The need for a more specific fluorescence technique was becoming evident. Investigations in this field were successfully conducted in this country and abroad. The results of one such rather thorough study, performed by a group from the Sanitary Centre of the USA Public Health Service, are presented below.

American scientists studied the luminescent properties of carcinogenic hydrocarbons that had been extracted from air

samples taken in large industrial cities. It was established that these compounds could be detected even in minute concentrations (10^{-9} g/ml). As a result, the most characteristic properties of polycyclic hydrocarbons were defined, which were later used as the basis for the development of specific methods for their identification.

Thus it was found, that among the 50 analogous compounds dissolved in concentrated sulphuric acid only 3,4-benzpyrene has a luminescence spectrum with a maximum at 548 nm. Because of this specific property, a quantity as small as 0.04 μg of 3,4-benzpyrene can be identified in a mixture of 50 analogous hydrocarbons, each of which is present in the amount of 1.0 μg (a total of 50 μg).

A group of Soviet investigators, working under the direction of L.M. Shabad, Member of the USSR Acad. Med. Sci., suggested a simple and elegant procedure for identifying 3,4-benzpyrene, based on the Shpolsky effect*. The substance under investigation was dissolved in hexane or octane, the resulting solution was frozen in liquid nitrogen, and the luminescence spectrum evaluated. The form of the spectrum

* The Shpolsky effect is the obtaining of thin-structured (consisting of separate bands) luminescence spectra by UV excitation of analyzed compounds in paraffin hydrocarbon solutions, after freezing them in liquid nitrogen (—196 °C).

provided a clear evidence as to whether 3,4-benzpyrene was present or absent in any particular solution.

All the methods for the identification of carcinogenic hydrocarbons described above, were immediately introduced as control measures for air pollution. It turned out that in industrial areas where the incidence of cancer is very high, and, in general, in large industrial cities with heavy traffic, 3,4-benzpyrene and analogous compounds were always found to be present in the air.

Thus, the exhaust gas is one of the main sources of carcinogenic agents released into the atmosphere. Indeed, of the 160 hydrocarbon derivatives that are components of industrial waste and exhaust gases, 20 are significantly carcinogenic. There can be no arguments about the paramount importance of studies directed at reducing the concentration of carcinogenic chemicals in exhaust gases. Today, specialists are recommending the following methods: (1) the stepwise burning of the products of incomplete combustion; (2) mixing of hydrocarbon fuel with hydrogen (the result will be a marked reduction in the concentration of organic compounds in the exhaust gases); (3) the replacement of the traditional spark ignition system in car engines with a system based on the torch ignition of air-fuel mixture. The latter method can reduce the concentration

of 3,4-benzpyrene in exhaust gases by 15-20 times.

In 1972, the USSR Ministry of Public Health established a limit for the concentration of 3,4-benzpyrene allowed in the air near industrial enterprises, as well as over towns and cities. These regulations are very strict: the concentration of 3,4-benzpyrene in the air near industrial area must not exceed 15 μg in 100 m^3, while in populated districts it must be 150 times less than that. This is half a million times lower than the limit set for another well-known air pollutant, sulphur dioxide.

After investigators began their serious search for 3,4-benzpyrene, the compound was found to be present practically everywhere. It was found, for example, in extracts of the ink used in newspaper printing, in smoked fish and sausage, in the lungs of city residents, and even in certain species of ocean mollusks that stick to the bottom of ships. In the last example, it was specifically proven that the hydrocarbons were of external origin, and not the products of the metabolism of mollusks.

The amounts of 3,4-benzpyrene found in printer's ink or in smoked food products are so minute that they are considered harmless; however concentrations of the carcinogen found in the lungs of addicted smokers are higher by tens and hundreds of times. Today it is obvious to everyone that smok-

ing drastically increases the incidence of bronchopulmonary cancer.

The carcinogenic effect of tobacco smoke can be seen only after many years of smoking; this is why many smokers do not believe in it. The World Health Organization has launched an anti-smoking campaign, and has appealed to the governments of all the countries of the world to take the necessary preventive measures. These recommendations include restrictions on smoking in trains, airplanes, buses, movie theatres, etc.

Of course, the most radical preventive measure is to stop smoking completely. However, only a very strong-willed person can succeed in this, especially after many years of regular smoking. Nicotine, the narcotic-like component of tobacco, makes it very hard for its victim to give up the harmful habit. The sarcastic comment by Bernard Shaw about how easy it is to give up smoking because he himself did so more than twenty times is well known and frequently quoted by smokers.

What advice can be given to those people who can not give up smoking? We will acquaint the reader with some of the recommendations to smokers suggested at the Second International Conference on Health and Tobacco, which took place in September 1971. These are: (1) do not inhale the cigarette smoke; (2) do not smoke the cigarette down to the end; (3) do not be too eager to

help yourself to somebody else's cigarettes; (4) buy cigarettes with low nicotine and resin content; (5) smoke only cigarettes with filters.

The author urges the readers to follow these recommendations.

Experimental studies on the physiological effect of tobacco smoke on animals have proven that carcinogenic agents are present in tobacco smoke. The incidence of malignant tumours was practically 100% in rabbits whose skin had been coated with the products of tobacco distillation. Another experiment demonstrated that otic cancer in the rabbit can be easily induced by blowing tobacco smoke through a special device into the floor of the auricle.

Fluorescence analysis has often been used to demonstrate the presence of carcinogenic hydrocarbons in tobacco combustion products. The concentration of 3,4-benzpyrene in cigarette smoke was quantitatively assessed in 1958. According to these data, when a person has smoked 100 cigarettes (five packages), approximately 0.5 μg of 3,4-benzpyrene, which is a considerable amount, enters his or her organism. Fortunately for smokers, a large part of this amount is soon removed from the lungs; otherwise, tumours would develop very quickly. Thus, fluorescence analysis has been employed in the struggle against smoking, which is one of the greatest hazards to one's health.

Conclusion

Medicine is one of the most ancient sciences, its history and traditions going back for many centuries. Fluorescence analysis, on the contrary, is a child of the twentieth century. It was introduced into medical practice only four or five decades ago. Medicine existed long before fluorescence analysis emerged, and would by all means have continued to exist without this method. But it would not be the modern medicine that we know today.

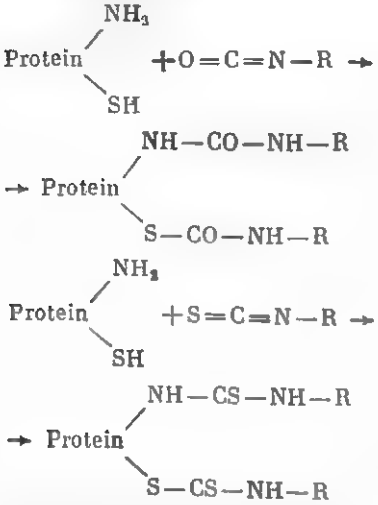
The amount of literature on the application of the fluorescence analysis has been increasing, but the actual practical application is still regretably limited. One of the possible reasons for this is the evidently limited effort to popularize the experience that has so far been accumulated.

This was in fact the motivation for writing this book.

Appendix

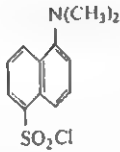
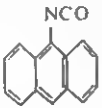
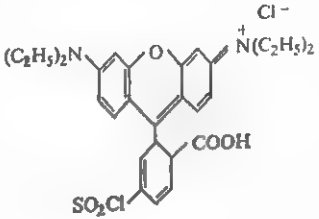
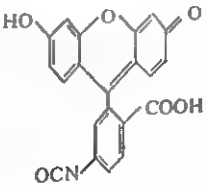
Proteins are usually labeled with stains containing sulfochloride, isocyanate, thioisocyanate and other groups within their structure. Reactive groups of the stains usually bind to the amino groups of the protein molecules, more precisely, the ε-amino groups of lysine residues. In addition, iso-

cyanate and thioisocyanate groups react with sulfhydryle protein residues:

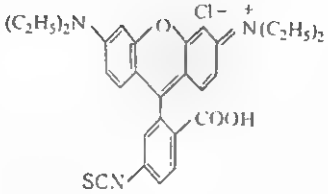
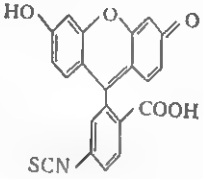
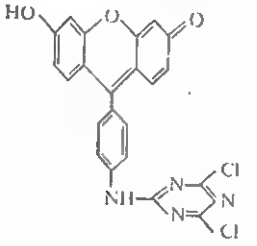


The structural formulas of some widely applied luminescent stains that form luminescent protein conjugates are given in the Table below.

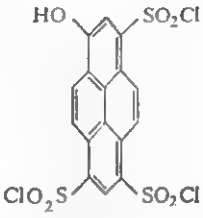
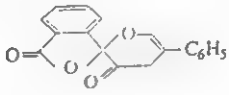
TABLE. Luminescent Stains for Labeling Proteins *

Name	Structural formula	Colour of emission
Dimethylamino-5-sulfonyl chloride		amber-green
9-Antryliso-cyanate		light blue
Rhodamin B sulfochloride		red
Fluorescein isocyanate		green

Continued

Name	Structural formula	Colour of emission
Rhodamin B isothiocyanate		orange-red
Fluorescein isothiocyanate		green
Dichlorotrianisinilaminofluorescein		green

Concluded

Name	Structural formula	Colour of emission
3-Oxypyrene-5,8,10-trisulfochloride		green
Fluorescamine		light bluish-green

* The optimal conditions for chemical binding of proteins by luminescent stains are dependent upon the type of the protein to be bound, and upon the chemical structure of the stain itself. For example, isocyanates (or sulfonylchlorides) and proteins are most reactive in thin water alkaline solutions (pH=7.5–8.5) at low temperature (0–4 °C). Reactions with isocyanates are best conducted in water-acetone solutions, at pH of 9.

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S. Yarmonenko

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